UNIVERSITEIT GENT

FACULTY OF **VETERINARY MEDICINE**
approved by EAEVE

Determinants in the pandemic H3N2 influenza A virus hemagglutinin that affect receptor binding and transmission in the pig

Sjouke Van Poucke

Dissertation submitted in fulfillment of the requirements for the degree of Doctor in Veterinary Sciences (PhD)

2013

Promotor: Prof. Dr. K. Van Reeth

Laboratory of Virology

Department of Virology, Immunology and Parasitology

Faculty of Veterinary Medicine

Ghent University

Van Poucke S. (2013). Determinants in the pandemic H3N2 influenza A virus hemagglutinin that affect receptor binding and transmission in the pig. PhD thesis, Ghent University, Belgium.

ISBN: 978-90-5864-345-2

About the cover: The influenza hemagglutinin structure is based on: Whittle J.R., Zhang R., Khurana S., King L.R., Manischewitz J., Goldin H., Dormitzer P.R., Haynes B.F., Walter E.B., Moody M.A., Kepler T.B., Liao H.X., and Harrison S.C. (2011). Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. PNAS 108: 14216–14221.

This study was funded by the EU Projects FLUPATH “Ecology and pathology of avian influenza virus” (project N° 44220) and FLUPIG “Pathogenesis and transmission of influenza in pigs” (project N°258084).

“It was never clear though whether the pigs were the culprits or the victims. Did we infect the pigs or did they infect us?”

Greger M. 2006

In: Bird flu: a virus of our own hatching. Lantern Books, New York City, USA

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

CHAPTER 1: GENERAL INTRODUCTION **13**

1.1. Introduction to influenza A viruses **14**
1.1.1. Classification and structure of influenza viruses 14
1.1.2. Influenza A virus replication cycle 16

1.2. Host range and ecology of influenza A viruses **17**
1.2.1. Birds 17
1.2.2. Mammals 19

1.3. The host range barrier **23**
1.3.1. Sialic acid receptor expression in the host and receptor specificity of the virus 24
1.3.2. The neuraminidase 31
1.3.3. PB2 amino acids 627 and/or 701 32
1.3.4. Binding of the polymerases to the nuclear import machinery 32
1.3.5. Decoy receptors 32

1.4. Cross-immunity between influenza A viruses from different species and/or other subtypes **34**

1.5. The pig as a" mixing vessel" for avian and mammalian viruses or as an intermediate host for adaptation of avian influenza viruses to a mammalian host **36**
1.5.1. The classical hypothesis 36
1.5.2. Arguments pro and contra 36

1.6. References **41**

CHAPTER 2: AIMS OF THE THESIS **59**

CHAPTER 3: REPLICATION OF AVIAN, HUMAN AND SWINE INFLUENZA VIRUSES IN PORCINE RESPIRATORY EXPLANTS AND ASSOCIATION WITH SIALIC ACID DISTRIBUTION **63**

CHAPTER 4: HOW A SWITCH IN RECEPTOR SPECIFICITY AFFECTS REPLICATION, TRANSMISSION AND CELL TROPISM OF A/HONG KONG/1/68 (H3N2) IN PIGS **91**

4.1. Effect of receptor specificity of A/Hong Kong/1/68 (H3N2) influenza virus variants on replication and transmission in pigs **93**

4.2. Does a switch in receptor binding specificity of A/Hong Kong/1/68 (H3N2) affect porcine respiratory tract cell tropism and interaction with mucus or surfactant proteins? **111**

CHAPTER 5: ASSESSMENT OF THE CONTRIBUTION OF HEMAGGLUTININ AMINO ACIDS 62, 81, 92, 144 AND 193 TO THE MAMMALIAN ADAPTATION OF A/HONG KONG/1/68 (H3N2) **137**

CHAPTER 6: GENERAL DISCUSSION **157**

SUMMARY-SAMENVATTING **177**

CURRICULUM VITAE **185**

DANKWOORD **189**

LIST OF ABBREVIATIONS

A	alanine
Aa	amino acids
AEC	3-amino-9-ethylcarbazole
AIV	avian influenza virus
AUC	area under the curve
BE	bronchial explant
CMC	carboxymethyl cellulose
CMI	cell-mediated immunity
CRD	carbohydrate recognition domain
cRNA	complementary RNA
CTL	cytotoxic T-lymphocyte
D	aspartic acid
DMEM	Dulbeco's modified eagle medium
dpc	days post contact
dpi	days post inoculation
E	glutamic acid
ECL	enhanced chemiluminescence
EID50	egg infectious dose with a 50% end point
ELISA	enzyme-linked immunosorbent assay
EMA	ethidium monoazide bromide
FITC	fluorescein isothiocyanate
G	glycine
Gal	galactose
H	histidine
HA	hemagglutinin
HAU	hemagglutinating units
HEPA	high-efficiency particulate air-filtered
HI	hemagglutination inhibition
HPAIV	highly pathogenic avian influenza virus
hpi	hours post inoculation
I	isoleucine
IF	immunofluorescence
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
IPMA	immunoperoxidase monolayer assay

K	lysine
L	leucine
LE	lung explant
LPAIV	low pathogenic avian influenza virus
M	matrix protein
MAA	*Maackia amurensis* agglutinin
MALDI-TOF	matrix-assisted laser desorption/ ionization time-of-flight
MDCK	Madin Darby canine kidney
MEM	minimum essential medium
mRNA	messenger RNA
N	asparagine
NA	neuraminidase
NE	nasal explant
NEP	nuclear export protein
NP	nucleoprotein
NS	non-structural protein
OD	optical density
PA	acid polymerase
PB	basic polymerase
PBS	phosphate-buffered saline
PFU	plaque forming units
Q	glutamine
R	arginine
RBS	receptor binding site
RNA	ribo nucleic acid
RNP	ribonucleoprotein
RT-PCR	reverse transcriptase polymerase chain reaction
S	serine
SD	standard deviation
Sia	sialic acid
SNA	*Sambucus nigra* agglutinin
SP	surfactant protein
SSE	secondary structural elements
T	threonine
$TCID_{50}$	tissue culture infectious dose with a 50% end point
TE	tracheal explant
TNF	tumor necrosis factor

Tr	triple reassortant
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
UP	ultra pure water
V	valine
VN	virus neutralization
vRNA	viral RNA
Y	tyrosine

CHAPTER 1.

GENERAL INTRODUCTION

1.1. Introduction to influenza A viruses

1.1.1. Classification and structure of influenza viruses

The three genera of influenza viruses: types A, B and C belong, together with the thogotovirus and isavirus, to the family of the *Orthomyxoviridae*. All members contain a segmented, negative-sense, single-stranded RNA genome that serves as a template for the generation of messenger RNA (mRNA) and complementary RNA (cRNA). The different influenza types A, B and C are distinguished by differences in the antigenic properties of the internal nucleoprotein (NP) and matrix 2 (M2) proteins, a different number of gene segments, host specificity (Table 1) and morphological characteristics of the virion (Wright and Webster 2001). Influenza A viruses, which are the topic of this study, are subdivided in subtypes based on the antigenic properties of hemagglutinin (HA) and neuraminidase (NA). To date, 17 different HA (Tong et al. 2012) and 9 different NA have been identified. Except for the H17N10 which has only been found in bats, they are all circulating in wild bird species (Dugan et al. 2008). The nomenclature of influenza viruses is based on the genus, the host of origin (for human isolates this is not mentioned), the geographical location of isolation, the strain reference number, the year of isolation and the antigenic subtype, e.g. A/Hong Kong/1/1968 (H3N2).

Table 1. Distinctive properties between different influenza types.

Type	Number of gene segments	Host tropism
influenza A	8	Humans, birds, horses, pigs, dogs, cats, marine mammals
influenza B	8	Humans
influenza C	7	Humans, pigs

The virion structure is pleiomorphic, including spherical and filamentous particles with a varying diameter between 80 to 120 nm (Harris et al. 2006). Three major components can be identified in every particle: an outer envelope, the matrix and 8 internal ribonucleoproteins (RNPs). The envelope is a lipid bilayer derived from the host cell membrane at the moment of virus budding containing both cholesterol enriched lipid rafts and non-raft lipids (Zhang et al. 2000). Three types of proteins are embedded in this membrane: the spike glycoproteins HA and NA, exclusively associated with the lipid rafts, and the ion-channel M2. HA makes up 80% of the envelope glycoproteins, NA about 17% and M2 a limited 3%. HAs are type-I integral membrane proteins composed of an N-terminal signal sequence, a membrane anchor domain and a short cytoplasmatic tail at the C-terminus. The HA is synthesized as a HA0 precursor protein that requires posttranslational cleavage into an HA1 and HA2 polypeptide, which are mutually connected by a disulphide-bridge. This modification can, depending on the pathogenicity of the virus, occur either extracellular by trypsin-like proteases (after the HA0

has been transported to the cell membrane) or intracellular by furin-like proteases in the trans-Golgi. The matrix 1 (M1) proteins form a layer underneath the envelope and interact with both the envelope and the RNPs. As such, they are responsible for the structural integrity of the virion and the encapsulation of the 8 RNPs (Sha and Luo 1997, Ruigrok 1998). The latter are composed of an RNA-segment, NPs and the polymerase complex PA, PB1 and PB2. A last structural protein, associated with the M1 protein, is the (misleadingly called) non-structural 2 protein (NS2) (also named the nuclear export protein NEP). Three non-structural proteins, being proteins that do not make part of the virus particle, also have been identified: NS1, PB1-F2 and PB1-N40. NS1 aides the virus to escape the anti-viral immune response of the host, especially the interferon α/β (IFN α/β) response, by binding dsRNA (Min and Krug 2006, Das et al. 2008, Hale et al. 2008). The PB1-F2, which is not expressed by all influenza A viruses, could play a role in promoting the apoptosis of monocytes and other immune cells (Chen et al. 2001, Conenello and Palese 2007). A single amino acid substitution (N66S) in the PB1-F2 of Hong Kong H5N1 (1997) and pandemic H1N1 (1918) was shown to contribute to pathogenicity in the mouse model. Viruses containing the mutation replicated to higher virus titers, induced elevated cytokine levels in the lungs and resulted in a lower survival rate (Conenello et al. 2007). These findings could not be extrapolated to other species such as pigs (Pena et al. 2012). The exact function of PB1-N40 has not yet been elucidated (Wise et al. 2009).

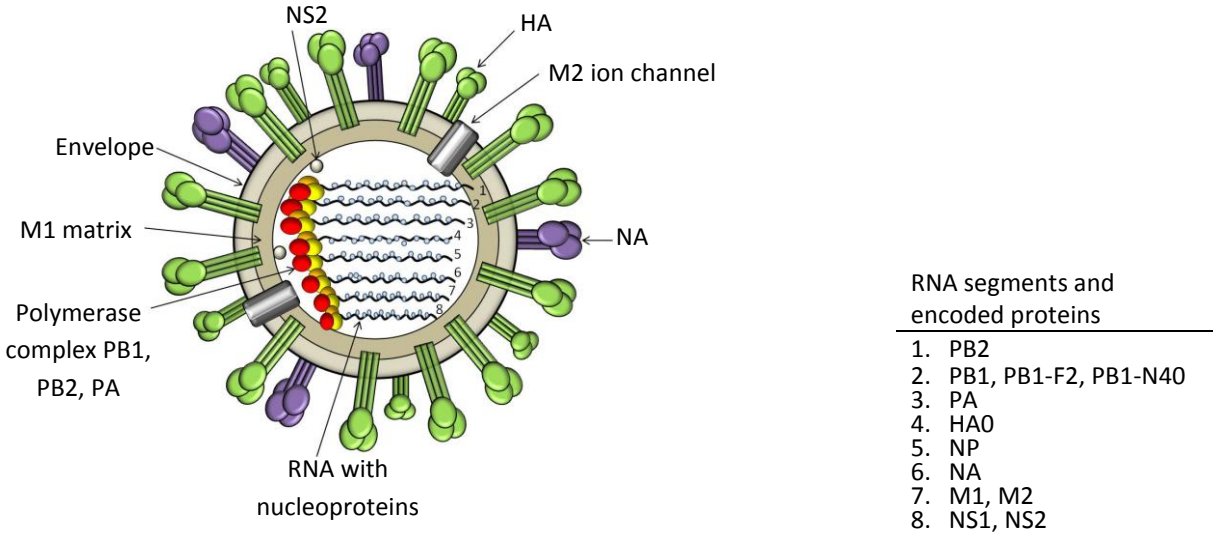

Figure 1. Schematic structure of an influenza A virion. The 8 gene segments encode for up to 12 proteins: basic and acid polymerases (PA, PB1, PB2), hemagglutinin precursor HA0, nucleoproteins NP, neuraminidase NA, matrix proteins M1 and M2, non-structural proteins NS1 and NS2 and finally the more recently discovered PB1-F2 and PB1-N40 (adapted from Lamb and Krug 2001).

1.1.2. Influenza A virus replication cycle

The entry of a virion into the host cell is initiated through the interaction of the receptor binding pocket at the globular head of the HA with sialic acids (Sia) at the cell surface. These Sia occupy terminal positions on oligosaccharide chains of glycoproteins or glycolipids. The low affinity individual contacts between Sia and HA are the result of hydrogen bonds, hydrophobic interactions and van der Waals contacts (Gamblin and Skehel 2010). Only through binding of multiple copies of Sia to multiple HA spikes, a high binding avidity is obtained. Adsorption of the virion is followed by clathrin mediated or clathrin and caveolin independent endocytosis (Skehel and Wiley 2000, Sieczkarski and Whittaker 2002, Lakadamyali et al. 2004). Migrating from the cell apex towards the nucleus in early and late endosomes respectively, the virus is preparing its escape from lysosomal destruction. The low pH environment of the endosome induces conformational changes in the HA, resulting in the protrusion of the hydrophobic HA2 fusion peptide and fusion of the viral envelope with the endosomal membrane (Whittaker and Digard 2006, Cross et al. 2009). Additional influx of protons through the M2 ion channel into the virus interior causes the disconnection of M1 proteins from the RNP, the uncoating of the virus and the release of the genome into the cellular cytoplasm (in the vicinity of the nuclear pore) (Whittaker and Digard 2006).

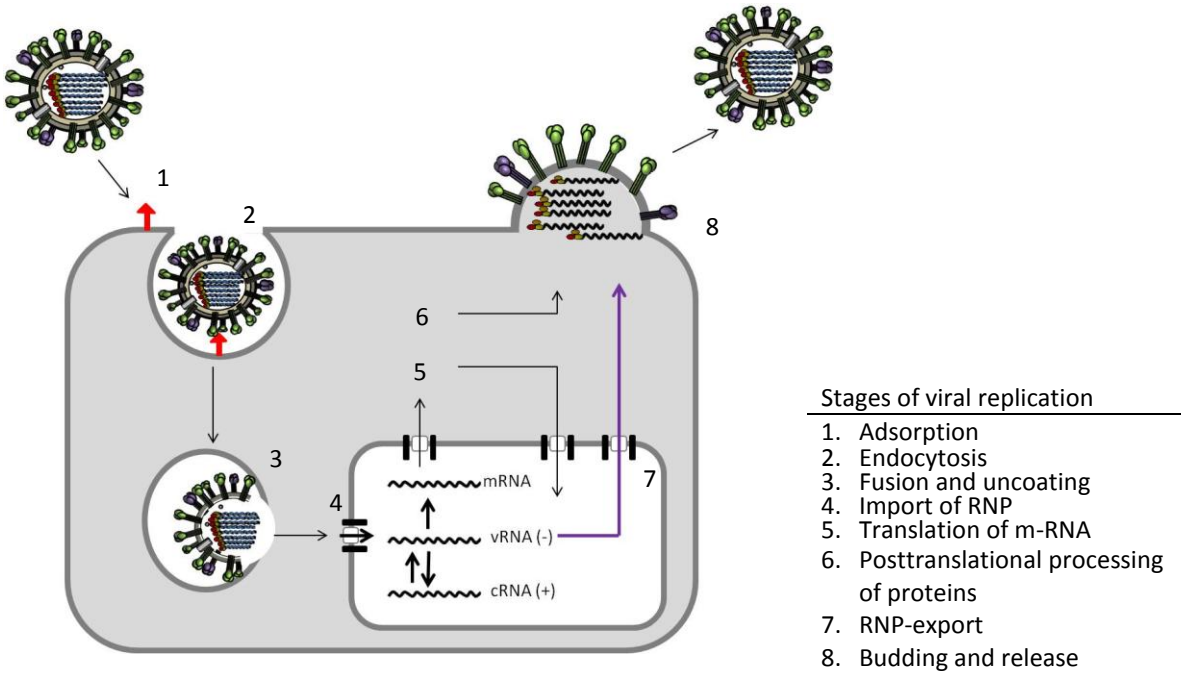

Figure 2. Schematic overview of the influenza A replication cycle (after von Itzstein 2007).

The active transport of the RNPs into the nucleus is mediated by binding of nuclear localization signals on PA, PB1, PB2 and NP to various karyopherins, such as importin α and β (Portela and Digard

2002). RNA transcription and replication take place in the nucleus. Transcription of viral RNA (vRNA) to messenger RNA (mRNA) is a complex event, as vRNA is missing the mandatory 5' methylated cap for this process (Das et al. 2010). The 5' cap is derived from host mRNA by a cooperative functional interaction of the polymerase complex. The endonuclease PA cleaves this cap from cellular mRNA and as such primes the transcription (Dias et al. 2009). Production of new vRNA requires the synthesis of an intermediate positive-sense template, called complementary RNA (cRNA). This cRNA serves as the template for the production of progeny RNA segments in the nucleus. All proteins comprising the RNP migrate from the site of synthesis in the cytoplasm to the nucleus where they associate with vRNA. The interaction of M1, NS2 and cellular nucleoporins mediates nuclear RNP export into the cytoplasm (O'Neill et al. 1998, Neumann et al. 2000). Virus assembly occurs through packaging of the genomic segments, NS2 and M1 underneath the apical cell membrane while HA, NA and M2 are already embedded in the latter. Budding of the virus is mediated by both M1 proteins and several host factors (Nayak et al. 2009). Finally the newly formed virion still needs to be released from the surface, where it is bound to Sia. The NA cleaves the Sia from glycoproteins and glycolipids and as such causes the final release of the particles (Gong et al. 2007).

1.2. Host range and ecology of influenza A viruses

Influenza A viruses infect a wide range of avian and mammalian species. Although humans, pigs, horses, dogs, felids, mink, ferrets and marine mammals all are natural hosts, the establishment of stable influenza lineages did not occur in the last 4 species.

1.2.1. Birds

Wild aquatic birds, particularly the orders *Anseriformes* (geese, ducks and swans) and *Charadriiformes* (gulls, terns and waders) are considered to function as the reservoir for 16 HA and 9 NA influenza genes (Stallknecht and Shane 1988). From all possible HA/NA combinations, at least 82 subtypes have been identified in these aquatic birds. Over 50% of the duck isolates belong to the H3-, H4- and H6-subtypes. The H13- and H16-subtypes are rarely found in birds other than gulls (Webster et al. 1998, Olsen et al. 2006). Replication of avian influenza viruses is restricted to epithelial cells lining the lower intestinal tract (from ileum to colon), the cloacal bursa and, to a lesser extent, the respiratory tract (Webster et al. 1978, Wood et al. 1995, Daoust et al. 2011). Because infections lead to no or mild clinical symptoms and high viral shedding in the faeces, perpetuation of the avian influenza viruses (AIVs) occurs efficiently by the fecal-oral route. The migratory behavior of these birds also allows intra- and/or intercontinental spread of the AIVs (Ito and Kawaoka 1998a, Fouchier et al. 2003). Since infections lead to low level humoral responses in these hosts, AIVs are exposed to a low population immunity pressure and a slow evolutionary rate. Still, based on phylogenetic

analyses, three distinct lineages can be distinguished: North American, Eurasian and gull viruses (Suarez 2000).

Influenza outbreaks in **domestic poultry** (such as chickens, turkeys, quails, commercial ducks and geese, pheasants and ratites) usually are the result of new introductions from feral birds. These viruses can be divided into two groups based on their ability to cause disease in chickens: high pathogenic (HP) and low pathogenic avian influenza viruses (LPAIVs). The virus pathotype is mainly associated with the presence or absence of polybasic amino acids at the cleavage site of HA0. HA is made up of 3 monomer HA0 precursors, which require posttranslational cleavage into the subunits HA1 and HA2 in order to become infectious. In the absence of the cleavage process, the protrusion of HA2 in the endosome does not occur. As a consequence the fusion of viral and cellular membranes is abrogated and the viral genome is not released into the cytoplasm, but destroyed instead. HPAIVs contain a series of basic amino acids at the cleavage site which are recognized by intracellular subtilisin-like proteases (e.g. furin). Since these enzymes are present in most eukaryotic cells it explains the virus' infectious character for a wide range of host cells. LPAIVs possess only a single arginine which is cleaved by trypsin-like proteases present in cells of the respiratory and intestinal tract (Steinhauer 1999, Alexander 2000, Post et al. 2012).

HPAIVs, also referred to as "fowl plague", always belong to H5- or H7-subtypes but not every H5- or H7-subtype (even if a multibasic cleavage site is present) is highly pathogenic. The viruses emerge after the introduction of a LPAIV into the poultry population. The high virulence is acquired through amino acid changes in the cleavage site, changes in the glycosylation pattern of HA and/or NA adaptations (Senne et al. 1996, Hulse et al. 2004). HPAIV infections are confined to poultry, except for the H5N3 infection in sterns (1961) and the H5N1 infections in waterfowl (2005) (Liu et al. 2005). Fowl plague outbreaks are associated with morbidity and mortality as high as 100%. Common clinical manifestations due to the systemic infection and the resulting virus localization in brain, heart, spleen, liver, pancreas, muscle, etc. are: depression, mucoid diarrhea, neurological symptoms, respiratory distress, sinusitis, drop in egg production, hemorrhages, cyanosis and oedema (Capua et al. 1999, Perkins and Swayne 2001, Woo et al. 2011).

In general, LPAIV infections result in no or mild clinical symptoms: depression, conjunctivitis, nasal discharge, drop in egg production and decreased food and water consumption (Morales et al. 2009, Spackman et al. 2010). Sporadically, also LPAIV have been detected beyond the respiratory and gastrointestinal tract (Toffan et al. 2008). Due to secondary bacterial infections, accommodation and

age influences, symptoms can become more severe (Franciosi et al. 1981, Mutinelli et al. 2003, Kishida et al. 2004, Elbers et al. 2005). Out of all susceptible species, turkeys seem most sensitive to infection (Tumpey et al. 2004).

1.2.2. Mammals

Figure 3, depicting "the wheel of influenza", clearly shows that all mammalian influenza viruses emerged through introduction of either an entire avian influenza virus or viral genes from the wild aquatic bird reservoir at some point. In humans and pigs similar prevalent influenza virus subtypes progressively adapted to the respective hosts, diverging into established lineages. A single introduction of an equine H3N8 influenza virus into dogs in the U.S.A. (around 2000) gave rise to the establishment of a new subtype that is currently still maintained in this host (Payungporn et al. 2008).

Species-specific lineages that have circulated in **humans** so far are confined to H1N1, H3N2 and H2N2 subtypes (Webster et al. 1992). As the overall population immunity in humans is rather high, human influenza viruses are forced to continuously adapt in order to overcome this pressure. As a result of successfully escaping the population immunity, two types of influenza outbreaks which are distinct in severity and geographical spread can be distinguished.

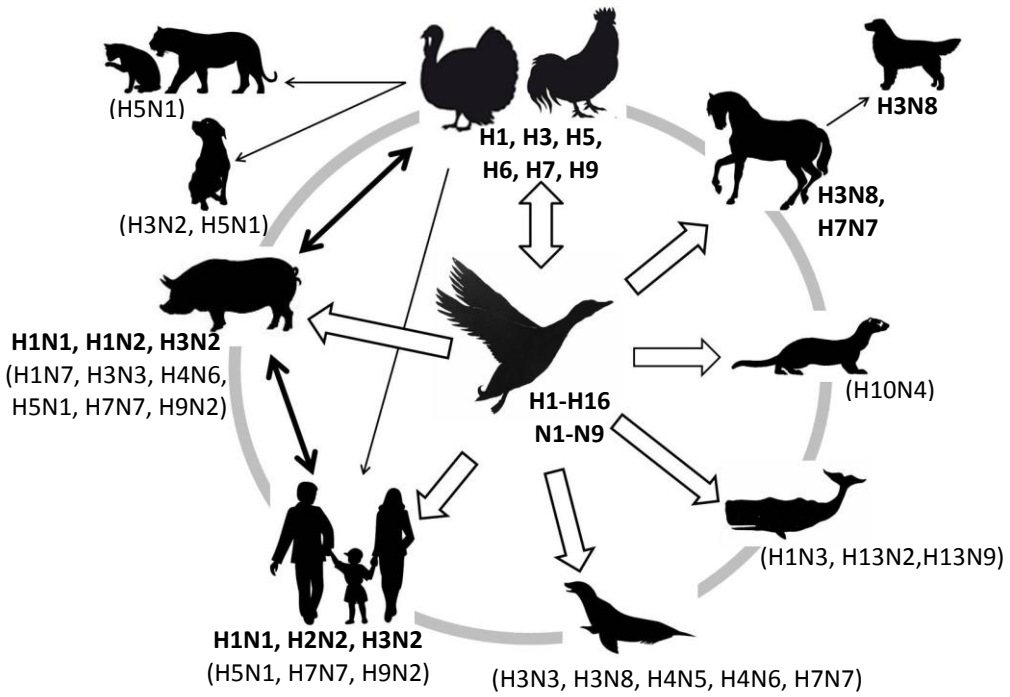

Figure 3. The natural hosts of influenza A viruses with centrally depicted the wild aquatic bird reservoir. The lineages established within the different hosts are in bold, the subtypes between brackets are occasional isolates (after Fouchier et al. 2003).

1) Epidemics refer to disease outbreaks affecting more than the expected number of people in a community or region during a given period of time. Annual/ seasonal epidemics in general are the result of *antigenic drift*. This type of adaptation is due to the accumulation of mutations in the surface glycoproteins HA and/or NA. RNA viruses intrinsically have a high mutation rate because their replicases and transcriptases lack proofreading-repair activities (Domingo and Holland 1997).

2) When an epidemic expands, spreading through the human population across a whole region, a continent or even the world it is called a pandemic. Several mechanisms, which are not mutually exclusive, can lead to a pandemic:

   - *Antigenic shift* refers to the introduction of a novel HA and/or NA to which the population has no immunity and such adaptations gave rise to the emergence of the majority of the human pandemics of the 20th and 21st century (an overview is shown in Figure 4). During this process 1 or both human adapted viral surface proteins are replaced by proteins from AIVs, also referred to as reassortment. This event occurs when the same cell is simultaneously infected with two different viruses allowing their gene segments to be exchanged. The Asian 1957 H2N2 and the Hong Kong 1968 H3N2 pandemics both resulted from the reassortment between the prevailing human viruses at that time and Eurasian avian viruses (Neumann and Kawaoka 2006).

   - *Introduction of an entire virus from a non-human host* (transmission *in toto*) is another possible mechanism. The Spanish 1918 H1N1 pandemic, is suggested to result from such an event. Although the composing genes are identified as avian–like, the exact source of this virus has not been pinpointed so far (Gorman et al. 1991, Taubenberger et al. 2005, Taubenberger and Morens 2006). Phylogenetic mapping studies however claim this pandemic was more likely generated by reassortment between strains circulating in humans or pigs and avian viruses over a period of several years (Smith et al. 2009b). The most recent 2009 H1N1 pandemic (pH1N1) resulted from an introduction of the virus from pigs, even though it was preceded by several reassortment events in this host (Gibbs et al. 2009).

   - *Reintroduction of an earlier circulating strain* that is hidden in a non-human reservoir until the human population immunity fainted has thus far only been reported once. The Russian

1977 H1N1 pandemic most likely was the result of such a reintroduction (Webster et al. 1992, Maines et al. 2008). Because the viral RNA of this H1N1 shows extensive similarities with strains prevalent in the 1950s, it is being speculated that this outbreak was due to an accidental release from a laboratory (Wertheim 2010).

Seasonal influenza infections are restricted to the human respiratory tract and are characterized by a high morbidity and low mortality. Zoonotic infections of humans with avian H9N2, HP H5N1, and LP/HP H7 viruses have repeatedly been reported as well (Peiris et al. 1999, Koopmans et al. 2004, Van Kerkhove et al. 2011). Symptoms caused by these infections vary from conjunctivitis and mild respiratory illness to a 60% mortality with pneumonia, multi-organ failure and acute respiratory distress. The severe outbreaks are particularly associated with HPAIV H5N1 (Peiris et al. 2004, de Jong et al. 2005, Korteweg and Gu 2008). Fortunately, these viruses so far lack the capacity of sustained human-to-human transmission and, therefore, have mainly led to dead-end infections (Wang et al. 2008, Peiris et al. 2009a).

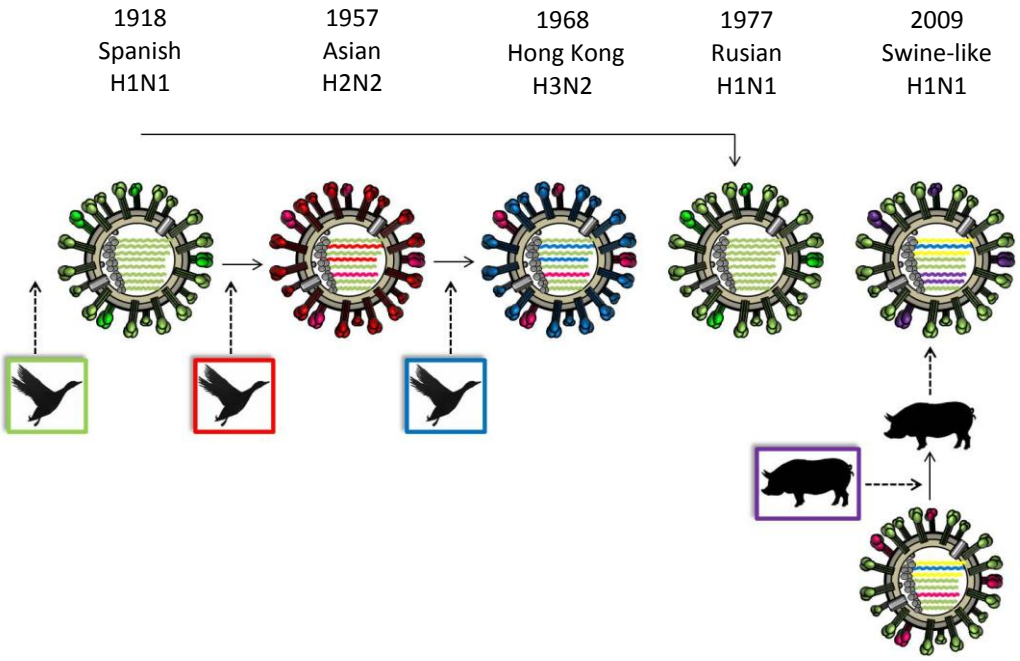

Figure 4. Chronicle of the evolution of the emergence of pandemic influenza A viruses in humans (after Sorell et al. 2011). The number of avian genes contributing to the pandemics of 1918, 1957 and 1968 were respectively 8, 3 and 2. The 2009 swine-like H1N1 (pH1N1) is a reassortant between the porcine American triple reassortant (Tr) virus H1N2 and the NA and M genes derived from an Eurasian porcine H1N1 virus (Smith et al. 2009a, Trifonov et al. 2009). The American Tr H1N2 contains PB1 and NA genes from human H3N2, PB2 and PA genes from an avian virus and the remaining genes from classical swine H1N1.

Pigs are susceptible to a wide range of influenza A virus subtypes but only H1N1, H3N2 and H1N2 circulate endemically worldwide (Guan et al. 1996, Brown 2000, Webby et al. 2000, Maldonado et al. 2006, Van Reeth et al. 2008, Kuntz-Simon and Madec 2009). Occasional isolations of other subtypes such as H1N7, H3N1, H3N3, H4N6, H5N1 have been reported from transiently infected pigs (Brown et al. 1997a, Karasin et al. 2000a, Karasin et al. 2004, Lekcharoensuk et al. 2006). Even when belonging to the same subtype, American, Asian and European strains differ both genetically and antigenically. In this paragraph we will only focus on the epidemiology of European strains, an overview is depicted in Figure 5.

In 1940 the first isolations of influenza viruses in European pigs took place. These viruses, belonging to the H1N1 subtype, were closely related to the early human H1N1 strains such as A/Puerto Rico/8/34. The initial reports in Europe of pigs infected with classical swine H1N1 (this virus has a common ancestor with the human pandemic H1N1 virus of 1918) date from the 1950s. After an absence from the population for an episode of 20 years this virus reappeared in Italy in 1976 and later on became endemic in Europe (Brown et al 1997b). In 1979 an avian-like H1N1 swine influenza virus (transmitted entirely from birds) started to circulate and eventually superseded the classical swine H1N1 (Pensaert et al. 1981, Scholtissek et al. 1983). Human-like H3N2 viruses (related to A/Hong Kong/1/68 and A/Port Chalmers/1/73) began infecting the European swine population in the early 1970s without causing disease outbreaks (Shortridge et al. 1977, Haesebrouck and Pensaert 1988, de Jong et al. 2007). Starting from 1984, this subtype suddenly displayed an epidemic character. Sequence analyses showed that this H3N2 contained 6 genes from the avian-like H1N1 and the remaining genes, encoding the surface proteins, from human-like H3N2 (Castrucci et al. 1993, Campitelli et al. 1997). The more recently emerged H1N2 subtype, first detected in the UK in the 1990s, turned out to have an even more complex gene composition. The HA gene was derived from a human H1N1, the NA gene from human-like swine H3N2 and the remaining genes from avian-like swine H1N1 (Brown et al. 1998, Marozin et al. 2002). Furthermore, new reassortant viruses between the three endemic swine influenza subtypes or between swine influenza viruses and seasonal human influenza viruses, have occasionally been detected in the last ten years and are still evolving (Hjulsager et al. 2006, Franck et al. 2007, Zell et al. 2008). Isolated outbreaks of infection with the pandemic H1N1 have also been reported in several pig herds in Europe (Hofshagen et al. 2009), as well as the world wide emergence of new reassortants between pH1N1 and endemic swine influenza viruses (Kitikoon et al. 2011, Starick et al. 2011, Liu et al. 2012).

Since most of the influenza subtypes mentioned above are endemically established, many herds harbor them without showing clinical symptoms. Only when immunological naïve pigs are infected the animals display typical symptoms associated with infection of the respiratory tract: fever, anorexia, lethargy, nasal and ocular discharge, coughing, sneezing and dyspnea. Outbreaks are characterized by a high morbidity, a mortality <5 %, a sudden onset and recovery within a week (Shope 1931, Jung et al. 2002, Van Reeth et al. 2012).

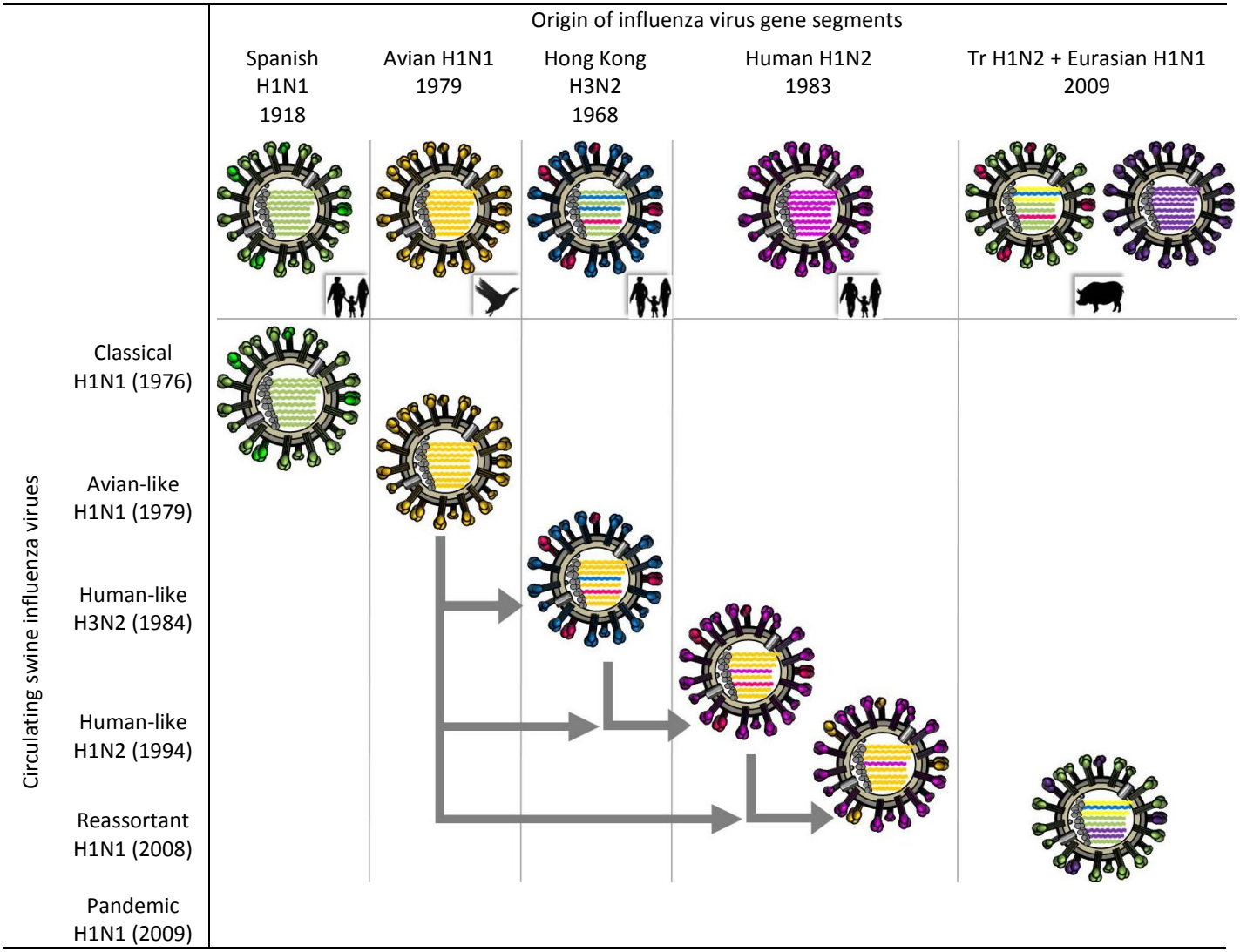

Figure 5. Chronicle of the evolution of the emergence of the major endemic influenza A viruses in European pigs.

1.3. The host range barrier

As can be seen from Figure 3, apart from virus transmission from wild aquatic birds to mammals (Bridges et al. 2002, Katz 2003, Anthony et al. 2012) and poultry also other interspecies transmission

routes take place: from pigs to humans and vice versa (Karasin et al. 2000b, Olsen et al. 2002b, Gaydos et al. 2006, Gray et al. 2007, Myers et al. 2007, Hofshagen et al. 2009), from pigs to poultry and vice versa (Choi et al. 2004, Nidom et al. 2010) and from poultry to humans. The relatively low frequency of interspecies transmissions and, moreover, the lack of subsequent persistence in a novel host, indicates that a strong species barrier exists (Kuiken et al. 2006). The host range restriction of influenza viruses is a complex and multigenic trait. Various presumed components of this barrier are discussed below.

1.3.1. Sialic acid receptor expression in the host and receptor specificity of the virus

The initial step of virus infection (adsorption) is established by an optimal interaction between terminal sialyloligosaccharides of glycoproteins or glycolipids on the target cells on one hand and the viral HA on the other hand (Skehel and Wiley 2000, Chu and Whittaker 2004). Based on the type of linkage between the carbohydrate and polypeptide fractions, glycoproteins are subdivided in N- and O-glycans. The latter typically contain shorter carbohydrate portions found in mucin-type glycoproteins (Hidari and Suzuki 2010).

a) Sialic acids (Sia) refer to a diverse family of negatively charged 9-carbon sugars which contain a pyranose ring with a carboxylic group, an amino group and a glycerol tail. The non-O-acetylated variants N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) are the most prevalently bound terminal receptor determinants. The latter are abundantly expressed in pig, horse, and mouse tissues but are absent in birds and humans. Extra diversity between Sia arises from one or several substitutions at the hydroxyl groups on the 4, 7, 8 and 9 carbons with acetyl, lactyl, methyl, sulfate, phosphate etc. (Varki 1997, Matrosovich et al. 2006). The type of α-glycosidic linkage between Sia and the penultimate oligosaccharide also displays variation and is one of the most determining factors for host specificity because they lead to different molecular shapes. The conformation of the Sia α2-3 linkage is linear (also referred to as trans conformation), meaning that the attached oligosaccharide is occupying a cone-like region. In contrast, the Sia α2-6 linkage in long glycans causes the sugars beyond the Gal to fold backwards (also referred to as cis conformation). As a result, the oligosaccharide spans a wider space on the HA surface and has an umbrella-like topology (Chandrasekaran et al. 2008). Figure 6 depicts a sterical comparison of both types of sialyloligosaccharides. The following combinations are recognized by influenza A viruses: α2-3 or α2-6 galactose (Gal), α2-3 or α2-6 N-acetyl-galactosamine (GalNAc) and α2-6 N-acetyl-glucosamine (GlcNAc) (Suzuki et al. 2000, Suzuki et al. 2001, Matrosovich et al. 2006, Nicholls et al. 2008). Even dissimilarities between more distal carbohydrate residues can selectively be recognized by viruses:

the nature of the third monosaccharide, the presence of fucose, sulfo- or Sia groups at this residue and β1-3 or β1-4 types of linkages (Yamnikova et al. 2003, Gambaryan et al. 2005).

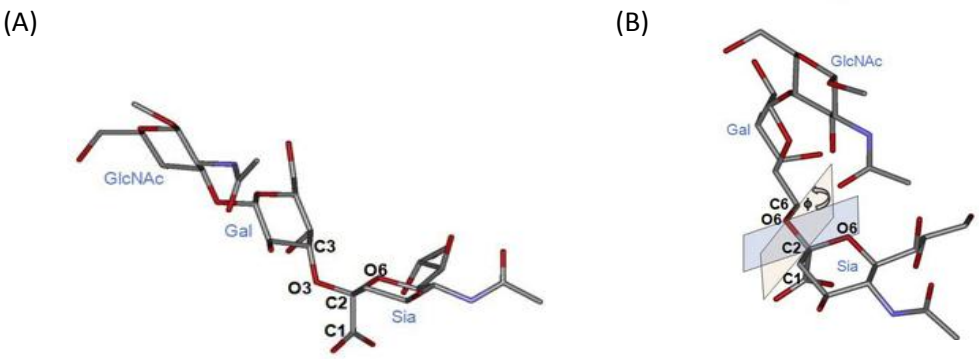

Figure 6. Conformational comparison of sialyloligosaccharides containing either an α2-3 (A) or α2-6 linkage (B) between Sia and the penultimate galactose (Gal). Panel (B) clearly shows that an α2-6 linkage gives rise to the folding back of the remaining oligosaccharide chain over the Sia, resulting in a more spacious structure (Jongkon et al. 2009).

The expression of the different receptor variants is host dependent and, within one particular host, also tissue and cell type dependent. The most commonly applied method to determine the receptor distribution pattern in tissue sections is by means of lectin histochemistry. Plant derived lectins such as *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA) are proteins that recognize and bind carbohydrates in a specific way. SNA is used to identify Sia α2-6 Gal linkages, while MAA (also called MAL) binds to Sia α2-3Gal linkages. Within the group of MAA lectins, two isoforms can be distinguished: MAA-I and MAA-II. They differentiate between β1-4 and β1-3 linkages connecting galactose with the next sugar, respectively. An overview of the receptor distribution in different species and tissues, based on literature, is shown in Table 2. The results obtained by different research groups sometimes appear to be contradictory. When interpreting lectin staining results, one has to keep in mind that variation between different lectin manufacturers occurs (Nicholls et al. 2007a), that host factors such as age, breed, infection status etc. can influence Sia expression (Yao et al. 2008, Kirkeby et al. 2009, Pillai and Lee 2010, Trebbien et al. 2011, Nicholls et al. submitted for publication) and that lectin binding is not 100% specific. Glycan array data have for example revealed that MAA-I will also identify non-Sia residues (Nicholls et al. 2008) and that cross-reactive binding of SNA with some Sia α2-3 receptors occurs. An alternative method to study glycans is by mass spectrometry. In the MALDI-TOF method (matrix-assisted laser desorption/ionization time-of-flight), molecules or atoms present in the sample firstly are turned into positively charged ions by exposure to a short laser pulse. These ions are then accelerated in an electric field generating an ion beam that passes a grid electrode. Subsequently, the time it takes for the ion to fly from one

end of the flight tube to the other end, hitting the detection plate, is measured. This time is proportional to the mass-to-charge ratio. Usage of specific sialidases that digest exclusively α2-3 (Sialidase S) or both α2-3 and α2-6 linkages (Sialidase A) will result in a shift of the generated profile and allows to obtain detailed information on the glycan variants present (Bateman et al. 2010). The disadvantage of this technique, when a piece of tissue is analyzed, is that no differentiation can be made between Sia present on the epithelium or those in submucosal tissues (which are unlikely to function as a receptor for influenza virus infection). Only by using laser-controlled microdissection could the epithelial cells specifically be separated.

Table 2. Distribution of sialic acid receptors in different hosts as reported by different research groups. Note the discrepancy between the results obtained by different researchers (and by different lectin suppliers), although all of these results are based on lectinhistochemistry.

Species	Tissues in which Sia….receptor variants are dominantly expressed on epithelial cells as determined by lectin histochemistry		
	α2-3	**α2-6**	Ref.
Duck	- intestines, trachea, bronchi, bronchioles, alveolar cells	- bronchi, bronchioles, alveolar cells	1
	- intestines, trachea, bronchi	- trachea, bronchi	3
Chicken	- intestines, bronchi, bronchioles, alveolar cells	- trachea, bronchi, bronchioles, alveolar cells	1
	- intestines, trachea, bronchi	- intestines, trachea	3
	- intestines (colon)	- intestines (colon)	4
Turkey	- intestines, trachea	- trachea	3, 5
	- intestines, trachea (non-ciliated cells)	- intestines, trachea (ciliated cells)	2
Quail	- intestines (colon)	- intestines (colon)	4
	- intestines, trachea	- trachea	5
Human	- bronchi, bronchioles, alveolar cells	- nasal mucosa, nasopharynx, trachea, bronchi, bronchioles, alveolar cells	6, 7, 8, 9
Pig	- trachea	- trachea	10,11
	- bronchioles, alveolar cells	- nasal mucosa, trachea, bronchi, bronchioles, alveolar cells	12, 13, 14

[1] Kuchipudi et al. 2009, [2] Wan and Perez 2006, [3] Pillai and Lee 2010, [4] Guo et al. 2007, [5] Kimble et al. 2010, [6,] Shinya et al. 2006, [7] Nicholls et al. 2007a, [8] Nicholls et al. 2007b, [9]Ito et al. 1998b, [10] Suzuki et al. 2000, [11] Punyadarsaniya et al. 2011, [12] Nelli et al. 2010, [13] Van Poucke et al. 2010, [14] Trebbien et al. 2011.

A last alternative to study Sia receptors is by means of virus histochemistry and determination of the pattern of viral attachment (PVA). Inactivated, directly labeled viruses are hereby used to display the attachment of these viruses to fixed tissue sections (Van Riel et al. 2007, Van Riel et al. 2010). This method only gives an indirect indication of the type of receptor that might be present, relying on the binding specificty of the virus that is used.

b) The binding activity of influenza viruses for different Sia-Gal linkage types is mainly determined by the membrane-distal portions of the HA trimers: **the receptor binding site (RBS).** This RBS, as depicted in Figure 7, is a shallow depression located in the globular head of HA1. The amino acid residues making up this RBS are in general located at similar amino acid positions, regardless the virus subtype that is studied. The major structural components are a bottom (amino acids (aa) 98, 153, 183 and 195), a rear (aa 190, 194 and 155), a right side (aa 134 until 138) and a left side (aa 224 until 228) (Skehel and Wiley 2000, Matrosovich et al. 2006, Matrosovich et al. 2008). Overall, the RBS of human influenza viruses interacts most efficiently with α2-6 linked glycans, the RBS of avian influenza viruses with α2-3 linked glycans whereas the RBS of pig influenza viruses can either equally bind both types or have a predominance towards α2-6 linked glycans (Connor et al. 1994, Sawada et al. 2010). A change in the receptor binding preference of an influenza virus due to mutations in the RBS is in most cases a prerequisite for breaking the species barrier. The type of residue mutations that occur may differ between HA subtypes or even within the same subtype (an overview is given in table 3).

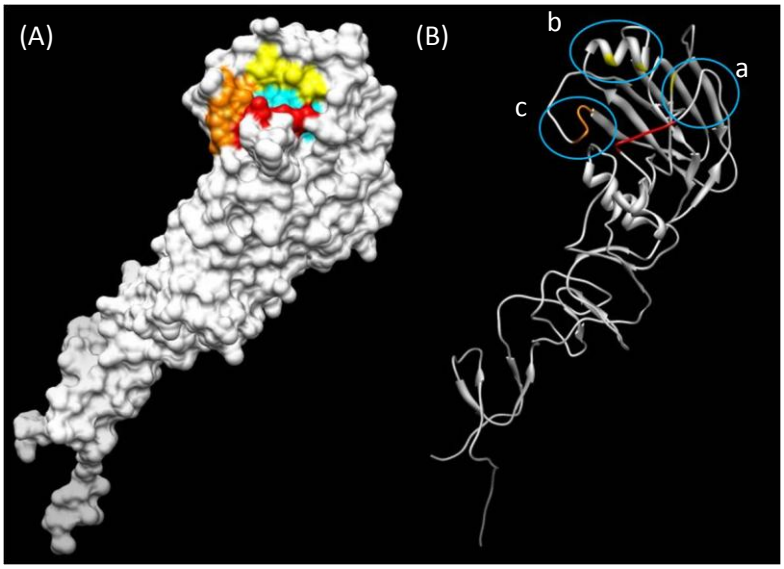

Figure 7. (A) Shows the surface representation of the receptor binding site in the HA1 monomer of A/Hong Kong/1/68 (H3N2). The central bottom made up by amino acids (aa) 98, 153, 183 and 195 is colored in cyan, the rear (aa 190, 194 and 155) in yellow, the right side in red (aa 134 until 138) and the left sidein orange (aa 224 until 228). In (B) the same HA1 is depicted as a ribbon representation, clearly visualizing some secondary

structural elements: the 130-loop (a), the 190-helix (b) and the 220-loop (c). The images were generated with the Chimera version 1.5.3. program.

As *in vivo* experiments have revealed that the correlation between a certain α–glycosidic linkage (α2-3 or α2-6) binding preference of a virus and its replication or transmission capacity in a host is not always perfectly matching, the hypothesis was raised that other viral components besides the RBS also contribute to the species specificity of an influenza virus. Tumpey et al. (2007) for example observed in a ferret model that pandemic 1918 H1N1 with a converted binding preference towards Sia α2-3 was equally virulent and efficiently replicating in the upper respiratory tract as the parental virus but lost its capacity of sustained transmission in ferrets.
Particularly with long Sia α2-6 linkage glycans, the viral HA is also interacting with more distal asialo-residues of the receptor. Therefore other viral features such as **secondary structural elements (SSE)** of the receptor binding domain and **β-strands** that form a trimeric interface can contribute to the fine tuning of the HA binding. The following SSE have been identified: 130-loop, 150-loop, 190-helix and 220-loop (Newhouse et al. 2009).

The antibody pressure to which (particularly human) influenza viruses are exposed can also indirectly influence their host range. The different antigenic sites of the influenza virus (sites A to D in H3N2 subtypes) are localized on the surface of the HA, some close to the RBS (Wiley et al. 1981, Whittle et al. 2011). One way to escape from antibody neutralization is by introduction of additional glycosylation sites in the HA so that antigenic epitopes are shielded. When this introduction occurs in the vicinity of the RBS, the receptor binding of HA will directly or indirectly be changed (either increased or decreased) (Ohuchi et al. 1997, Vigerust et al. 2007). Hensley et al. (2009) on the other hand, reported the identification of HA mutations in the globular head domain that increased the viral HA binding avidity for cell surface receptors in respons to escaping polyclonal antibodies.

Under *in vitro* conditions, Daniels et al. (1987) demonstrated for the first time that amino acid substitutions located proximally to the RBS also can influence the pH at which membrane fusion occurs. Recent experiments in ferrets have provided evidence that the pH threshold for fusion activity indeed can influence the HA protein stability and the transmission efficiency of a virus. Imai et al. (2012) selected reassortant viruses based on their capacity to bind Sia α2-6, containing mutated HA genes from HP avian H5N1 virus and the 7 remaining genes from pH1N1. They concluded that efficient transmission in ferrets only took place when the virus, in addition to Sia α2-6 binding, also had a mutation (T318I) in the vicinity of the fusion peptide reducing its fusion threshold to a pH 5.5.

Table 3. Amino acid substitution(s) in avian HAs, belonging to different influenza virus subtypes, resulting in the adaptation to a mammalian host. The following amino acids are highly conserved in avian viruses: 138A, 190E, 194L, 225G, 226Q and 228G (Matrosovich et al. 2000).

Virus subtype	Host of isolation	Amino acid substitution(s) from an avian-like towards mammalian-like HA	Effect of the amino acid substitution on the virus phenotype	Type of outbreak (in human)	Ref.
H3N2 (1968)		R62I D81N N92K N/A144G V182I N193S Q226L G228S	? ? ↑ Interaction with oligosaccharides ↑ Sia α2-6 binding ↓ Sia α2-3 binding	P	13 10, 11, 12
H2N2 (1957)		R137Q G158E N186I Q226L G228S		P	11, 12
H1N1 (1918)		T159G E190D G225D	? ↑ Sia α2-6 binding, ↓ Sia α2-3 binding	P	7, 8, 9, 11
Avian-like swine H1N1 (1979)		T155I/V T159N E190D G225D	↑ Neu5Gc binding ? ↑ Sia α2-6 binding, ↓ Sia α2-3 binding	E	11 15
Avian H4N6		Q226L G228S	↑ Sia α2-6 binding, ↓ Sia α2-3 binding	I	3
HPAIV H5 in pH1N1 background		N158D N224K Q226L T318I	Loss of glycosylation site ↑ Sia α2-6 binding, ↓ Sia α2-3 binding ↓ the pH treshold for fusion activity	L	1
HPAIV H5N1		H107Y T160A/ N158K Q226L G228S	↑ Sia α2-3+ Sia α2-6 binding Loss of glycosylation site, ↑ Sia α2-3+ Sia α2-6 binding ↑ Sia α2-6 binding	L	2,14
H9N2		A190T Q226L	↑ Sia α2-6 binding, ↓ Sia α2-3 binding	L	4, 5, 6

[1] Imai et al. 2012, [2] Herfst et al. 2012, [3] Bateman et al. 2008, [4] Matrosovich et al. 2001, [5] Wan et al. 2008, [6] Wan and Perez 2007, [7] Matrosovich et al. 1997, [8] Glaser et al. 2005, [9] Tumpey et al. 2007, [10] Vines et al. 1998, [11] Matrosovich et al. 2000, [12] Connor et al. 1994, [13]Bean et al. 1992, [14] Gao et al. 2009, [15] Dunham et al. 2009

Nonpolar hydrophobic amino acids: G: glycine, A: alanine, V: valine, L: leucine, I: isoleucine

Polar hydrophilic amino acids: S: serine, T: threonine, Y: tyrosine, N: asparagine, Q: glutamine

Negatively charged amino acids: D: aspartic acid, E: glutamic acid

Positively charged amino acids: K: lysine, R: arginine, H: histidine

P: pandemic outbreaks, E: endemic outbreaks, L: lab strain, I: individual case

c) Whether a particular influenza virus will or will not bind to a certain receptor depends on the interaction between all factors described under a and b. Here, several methods to determine the affinity of influenza viruses for different receptor analogues are described. One of the oldest techniques relies on the use of red blood cells (RBC) expressing defined receptor variants. These are either natural RBC obtained from different species (Ito et al. 1997) or resialylated RBC. The latter RBC are first pretreated with *Vibrio cholerae* neuraminidase to remove all Sia and are subsequently resialylated by applying specific sialyltransferases (Paulson and Rogers 1987). These RBC are used in a virus hemagglutination or hemadsorption assay. Later, a more sophisticated competitive fetuin binding inhibition assay was developed. This test is based on the inhibition of binding of a standard preparation of horseradish peroxidase labeled fetuin to solid-phase immobilized virus by different sialylglycopolymers. Fetuin is a naturally occurring glycoprotein that possesses both Sia α2-6 and Sia α2-3 linkages (Gambaryan and Matrosovich 1992, Gambaryan et al. 1997). In a more direct variant of this test, the binding of biotin labeled sialylglycopolymers to solid-phase immobilized viruses is assayed.

Meng et al. (2010) have used surface plasmon resonance in order to detect the amount of adsorption of purified viruses to a sensor chip coated with fetuin. The purified viruses were preincubated with either free 3'-sialyllactose or 6'-sialyllactose. The higher the binding affinity of a virus for a particular free sialyllactose, the fewer viruses remained to bind to the fetuin.

Glycan arrays belong to the most recent and most detailed techniques to determine the differential receptor binding affinities of influenza A viruses. The binding of either entire influenza viruses or recombinant produced HA to a wide array of glycan variants, that are bound to a glass slide, is detected by fluorescent antibodies (Stevens et al. 2006b, Liao et al. 2010). Using recombinant HA proteins instead of entire virions circumvents possible interference of the NA activity on the binding assay. Thanks to the wide range of imprinted glycoproteins, also fine differences in HA specificity such as fucosylation, sulfation and sialylation at positions 2 and 3 of the terminal trisaccharide can be assessed (Stevens et al. 2006a).

1.3.2. The neuraminidase

NA, the second spike glycoprotein on the surface of influenza viruses, has a tetramer conformation and an enzymatic activity. By hydrolysis of the α-ketosidic linkage it enables the removal of terminal Sia from oligosaccharides. In this way, it helps the release of progeny virus, the prevention of self aggregation and the escape from extracellular inhibitors (Colman 1994). Multiple mechanisms by which it affects host specificity have been identified.

The interplay between the receptor-binding function of HA and the receptor-destroying function of NA needs to be in balance. Similar to HA, NA also has a substrate preference for particular α–glycosidic linkage variants. It was demonstrated that the NA of human, avian and swine isolates have a similar and preferential specificity for Sia α2-3 linkages but that the activity against Sia α2-6 linkages ranges from marginal to highly efficient in avian and human/pig isolates respectively (Couceiro and Baum 1994, Kobasa et al. 1999). In addition, NA also distinguishes substrate structures at the inner part of the oligosaccharide (Mochalova et al. 2007). By passaging reassortant viruses with avian HA and human NA (derived from various subtypes) Shtyrya et al. (2009) have shown that replication was reconstituted when mutations occured in HA and/or NA which lead to a functional balance.

It was, furthermore, reported that avian influenza viruses and early isolates of the pandemic 1957 H2N2 and 1968 H3N2 isolates, in contrast to human and swine viruses, retained their neuraminidase activity even under low pH conditions (pH < 4.5). This characteristic would allow the viruses to maintain their infectivity after passage through the digestive tract and seems to correlate with the specific enterotropism of avian influenza viruses (Takahashi et al. 2001). Besides a role in replication potential and tissue tropism, the HA-NA balance has also been suggested to influence transmissibility. An example is provided by the emergence of the pandemic H1N1 2009. As described under 1.2.2, this virus is the result of reassortment between avian-like Eurasian (EA) H1N1 and triple reassortment (Tr) H1N2. Yen et al. (2011) demonstrated that the respiratory droplet transmission of the individual precursors of the pandemic virus, belonging to the Tr or EA swine lineages, did not occur in ferrets. Only after introduction of the pandemic NA into sw915 (this is a Tr with the M gene derived from EA H1N1) a gene constellation that conferred efficient transmission was obtained. Likewise the substrate specificity of NA of the pandemic H2N2 1957 acquired an increased affinity for Sia α2-6 over time through amino acid substitutions.

Besides the enzymatic activity of NA, the length of the stalk region also has some influence on the host specificity. Adaptation of AIV from wild birds to gallinaceous or mammalian hosts is commonly associated with amino acid deletions in the stalk region (Banks et al. 2001, Chutinimitkul et al. 2010, Li et al. 2011).

1.3.3. PB2 amino acids 627 and/or 701

For several influenza virus subtypes including human isolates of HPAI H5N1, H3N2, H1N1 and avian isolates of H2N2, H7N7 and H4N6, the amino acids at the locations 627 and/or 701 of the PB2 have been identified to contribute to host specificity (Gabriel et al. 2005). Mammalian viruses typically contain lysine (K) and asparagine (N) at these locations whereas avian viruses possess glutamic acid (E) and aspartic acid (D) respectively. An influenza virus with an "avian-like" PB2 cannot replicate efficiently at 33°C, the temperature of the human upper respiratory tract, while a temperature of 37°C becomes permissive (as would correlate with the higher body temperature of birds). Also the transmissibility between mammals of such a virus, so far studied in guinea pigs and ferrets, is hampered (Matrosovich et al. 2009, Steel et al. 2009, Van Hoeven et al. 2009). *In vitro* studies showed that an E at 627 results in a decreased association of PB2 with NP in mammalian cells (Subbarao et al. 1993, Massin et al. 2001) while N at 701 enhances the binding of PB2 to importin α1 and thus the nuclear localization of the polymerase in mammalian cells. Another study by Scull et al. (2009) however indicated that the presence of E 627 resulted in a replication restriction of the virus, regardless the temperature condition (32°C versus 37°C).

However, the recent pandemic 2009 H1N1 has shown us that this so-called virulence marker is not an absolute feature of mammalian-adapted viruses. The PB2 of pH1N1 belongs to the avian-like type and mutations in one or both amino acids towards the mammalian-like type did not increase the virulence nor the transmissibility of this virus in mice or ferrets (Herfst et al. 2010). Yet, Yamada et al. (2010) reported that replacement of glutamine 591 with lysine may compensate for these avian-like sequences.

1.3.4. Binding of the polymerases to the nuclear import machinery

As mentioned under 1.1.2, the import of the polymerase complex and NP into the nucleus is an essential step of the influenza virus replication cycle. However, this process varies between the different polymerase subunits. The PB1/PA dimer is imported through binding of RanBP5 (= importin 5) while PB2 and NP are imported separately via importin α1/α5 and importin α1/α2 respectively (Watanabe et al. 2010). Gabriel et al. (2008) have shown that mouse adaptation of an avian PB2 and NP through single amino acid mutations resulted in higher binding with importin α1 and a more efficient import of the proteins into the nucleus in mammalian cells but not in an avian cell line.

1.3.5. Decoy receptors

Decoy receptors refer to a group of innate immune defense molecules, particularly important in the early stages of infection, that hamper interaction of influenza viruses with their target cells in

different ways. These molecules can directly block the receptor-binding sites of HA, create steric obstacles or cause aggregation of virus particles. By opsonisation of the viruses they can also modulate the activity of alveolar macrophages and neutrophils.

The respiratory tract, starting from the nasal mucosa down to the bronchioles, has an epithelial lining covered with **mucus** (Kim et al. 2011). The mucus is secreted by goblet cells as well as submucosal glands. The composition of the mucus layer is biphasic: an aqueous sol layer on top of the cell glycocalyx covered by a superficial gel layer. The latter is made up of high molecular weight glycoproteins, called mucins, linked with proteins and lipids. Apart from the dual structure, also the functions of the two layers are distinct. The sol layer provides a suitable environment for the beating of the cilia so the mucociliairy transport can carry the mucus (including captured viruses) towards the pharynx. Since the mucins of the gel phase are heavily glycosylated (mainly 0-glycans), they can compete with the cell receptors for the binding of viral HA (Couceiro et al. 1993, Scharfman et al. 1995, Bansil and Turner, 2006). Once more the functional balance between the HA and NA activity is supposed to determine how efficiently the viruses can detach from the mucus and reach the target cells after all. In human tracheobronchiolar epithelial cell cultures, Matrosovich et al. (2004a) observed a clear reduction in infectivity by blocking the enzyme activity of NA with oseltamivir carboxylate. Since the number of infected cells were counted at 7 hours post inoculation (hpi), they concluded that the reduced infectivity was due to a hampered process early in infection, such as the penetration of the mucus layer. Because the degree of reduction was stronger for heterologous LPAI viruses than for the homologous human strain studied, this seemed to confirm the antiviral potential of mucus. More recent work by Roberts et al. (2011) applied another assay to screen the antiviral activity of mucus against two viruses that differed only by L226Q and S228G mutations in the HA and that had their receptor specificity converted to avian-like. Their experimental data are not in line with the earlier hypothesis. These authors incubated the viruses with nasal ferret washes for 1 hour and assessed the percentage of remaining infectious particles by plaque titration of these mixtures on Madin Darby canine kidney (MDCK) cells. SNA and MAA histochemistry on the nasal washes additionally showed the abundant presence of Sia α2-6 Gal linkages. Surprisingly the wild type virus was more efficiently blocked than the avian-like variant. They suggested that a stronger binding of influenza viruses with the mucus might be beneficial by preventing the virus from being expelled with the mucociliary transport.

Surfactant proteins A and D (SP-A, SP-D) belong to the lung collectins and are characterized by a similar composition: an N-terminal region, a collagen-like domain, a neck and a Ca^{2+} dependent carbohydrate recognition domain (CRD). Although both proteins are produced by type 2

pneumocytes and non-ciliated bronchiolar epithelial cells (Clara cells), they differ in morphology and in the way they interact with influenza A viruses. The α2-3 and α2-6-linked Sia residues present on an oligosaccharide moiety of SP-A CRD bind to the RBS of HA. In contrast, SP-D interacts directly (and in a CA^{2+} dependent manner) via its CRD with N-linked high mannose carbohydrates of HA and NA (Matrosovich et al. 2003, van Eijk et al. 2004). As a consequence, the inhibitory capacity of SP-D against influenza viruses with a poorly mannose glycosylated HA is inefficient. Porcine SP-D, however, was shown to have distinct structural features compared to human, bovine, mouse and rat SP-D, including a cysteine in the collagen-like domain, 3 amino acid insertions in the CRD and the presence of terminal linked Sia in the CRD. Overall, porcine SP-D was shown to have a higher activity and a broader range of interaction with various strains compared to any of the other species studied (van Eijk et al. 2003). Since the terminal linked Sia in the CRD are exclusively α2-6-linked, one might assume that viruses with this type of binding preference are more likely to be impeded. This was confirmed in a recent study by Hillaire et al. (2011a) in which 30 viruses of avian, swine and human origin belonging to either H1N1, H3N2 or H5N1 subtypes were subjected to an infection reduction assay. The pig H3N2 for example was 84,9% inhibited, while the human and avian isolate 72,9% and 52,3% respectively. It remains puzzling how these findings can fit in *in vivo* observations where viruses with an α2-6-binding preference are more infection competent in pigs.

1.4. Cross-immunity between influenza A viruses from different species and/or other subtypes

Upon infection of an animal with influenza viruses, two types of responses are triggered: the innate and the adaptive immunity. Both types include humoral and cellular components but differ in the promptness, the specificity of induction and the generation of immunologic memory (Tamura and Kurata 2004). The **innate immunity** is a non-antigen specific, early induced, first-line defense mechanism lacking the capacity to induce a memory. Pattern recognition receptors (PRR) on sentinel cells such as dendritic cells (DC's), macrophages, natural killer cells (NK), neutrophils, γδ T-lymphocytes and epithelial cells will bind unique conserved viral sequences. The PRR, designated toll-like receptors (TLR), nucleotide-binding domain and leucine-rich-repeat receptors (NLR) and retinoic acid-inducible gene-I like receptors (RLR), are expressed in different cellular compartments (Joshi et al. 2009, Kawai and Akira 2009, Peiris et al. 2009b). Stimulation of this branch of the immunity results in the release of effectors, comprising diverse proinflammatory cytokines (interferons-α, -β and -γ, interleukins-1, -6 and -12, tumor necrosis factor), chemokines (IP-10, IL-8 and RANTES), complement and acute phase proteins (Van Reeth et al. 2002, Barbé et al. 2011). Findings by Zhou et al. (2006) suggested that the extent of chemokine responses and chemokine receptor expression in monocyte derived human macrophages differs between human and avian influenza viruses. Ramos and

coworkers (2011) showed that a virus exclusively differing by a higher α2-3 receptor specificity and independent from the extent of virus replication, could induce higher levels of innate immune responses in human primary DC's, macrophages, and respiratory epithelial cells. Although this observation seems to correlate with the hypercytokinemia observed in fatal cases of human infections with HPAI H5N1 (de Jong et al. 2006), the exact influence of receptor binding properties on virus virulence by this mechanism has not yet been elucidated.

Cross-protection, irrespective of whether it is heterosubtypic (against an influenza virus of another subtype) or subtype-specific (against a drift virus within the same subtype), is due to the **adaptive immunity** which is capable of developing a memory (Tamura and Kurata 2005, De Vleeschauwer et al. 2011). The humoral component, produced by activated B-cells, comprises specific antibodies at systemic or respiratory mucosal sites mainly directed against surface glycoproteins HA and NA. Antibodies against more conserved M1, M2, NS1, NS2 and NP are also produced but to a lesser degree (Larsen et al. 2000, Kim et al. 2006). By binding or covering the RBS, anti-HA antibodies can neutralize the virion, while anti-NA antibodies restrict the release of the virus after budding from the host cell. These antibodies only provide cross-protection between heterosubtypic isolates if their antigenic epitopes display enough similarity (Subbarao et al. 2006, Gillim-Ross and Subbarao 2007, Van Reeth et al. 2009). Heinen et al. (2001) provided indications that anti-M2 antibodies in pigs can also be involved in heterosubtypic immunity (swine H1N1 infected pigs challenged with swine H3N2) as this protein is antigenically conserved. Similar observation were made in mice and ferrets (Fiers et al. 2009).

Activation of the cellular immunity requires presentation of major histocompatibility complex (MHC)-I or MHC-II associated antigens to CD8+ or CD4+ T-cells. Upon stimulation, CD8+ T-cells differentiate into cytotoxic T-cells (CTLs) which release cytokines (interferon-γ, tumor necrosis factor-α) and perforines. Differentiated CD4+ T-cells, being either T-helper 1 or 2 cells, can act cytolytic or support B-cell and CTL activity. Mainly CTLs recognizing NP, PA, PB2 and M antigens have been proven essential for heterosubtypic immunity although CD4+ T-cells are contributing as well (Heinen et al. 2001, Subbarao et al. 2006, Hillaire et al. 2011 b).

1.5. The pig as a" mixing vessel" for avian and mammalian viruses or as an intermediate host for adaptation of avian influenza viruses to a mammalian host

1.5.1. The classical hypothesis

The contributions of AIV genes to the emergence of new human influenza viruses, as described under 1.2.2., have been accepted by the scientific community without much dispute. Yet, how and where reassortment between avian and human influenza viruses or the adaptation of AIV to a mammalian host occurs, have since long been a topic of debate. Experimental inoculations of human volunteers with AIV belonging to H1N1, H3N2, H4N8, H6N1 and H10N7 subtypes by Beare and Webster (1991) after all had shown that humans were not readily infected with AIV. Consequently, the suggestion was raised that another animal species, susceptible to avian and human influenza viruses, could function either as a "mixing vessel" or as an "intermediate host". Based on the rescue of a temperature sensitive NP mutant of A/FPV/Rostock/1/34 (H7N1) by double infections of chicken embryo fibroblast cells with either avian, human or swine H3N2 isolates, Scholtissek et al. (1985) proposed that pigs would be a suitable candidate. They found that the virus could be rescued by all AIV, by none of the human influenza viruses and by 2 out of 10 swine influenza viruses, indicating that the NP of swine influenza viruses has a broader host range regarding its compatibility in reassortant viruses. Later on, this hypothesis was strengthened by Kida et al. (1994) and Ito et al. (1998b). They, respectively, demonstrated that at least one avian strain of each HA subtype can replicate in pigs to levels similar as swine viruses and that the porcine trachea contained both α2-6 and α2-3 linked Sia.

1.5.2. Arguments pro and contra

Numerous natural (Karasin et al. 2000a, Karasin et al. 2004) and experimental (Kida et al. 1994, De Vleeschauwer et al. 2009a, 2009b) infections have confirmed that pigs indeed support replication of a wide range of AIVs. More detailed studies, however, have revealed that the productive replication of most of these viruses is significantly lower than that of swine adapted viruses, especially in the upper respiratory tract. This was particularly clear in a comparative pathogenesis study of an avian H5N2 and swine H1N1 after intratracheal or intranasal inoculation by De Vleeschauwer et al. (2009a). Although AIV infected fewer cells than swine viruses at all levels, infected cells were most scarce in the respiratory part of the nasal mucosa regardless of the inoculation method used. These findings were confirmed by experiments in *ex vivo* porcine respiratory tract systems derived from the respiratory part of the nasal mucosa and the trachea (Van Poucke et al. 2010, Löndt et al. 2012). The individual susceptibility of pigs to AIVs furthermore is highly variable. Inoculation of a group of

animals can result in a lack of infection in one pig and high virus titers in another pig. Moreover, identical influenza subtypes isolated from different hosts, can display pronounced differences in infection outcome in pigs. Lipatov et al. (2008) for example studied HP H5N1 AIVs isolated from humans, ducks, chickens and swans belonging to the major phylogenetic and antigenic clades. They detected nasal virus excretion in all pigs inoculated with the swan isolate and in none of the animals inoculated with the duck strain. Overall, the susceptibility of pigs to infection with all of these variants was lower compared to that of ferrets and mice. Very recent studies by Nelli et al. (2012), using human and swine respiratory epithelial cells and macrophages, showed that the pig cells had no or weak TNF-α and chemokine induction after HPAI H5N1 infection, resulting in a greater cell death and a reduced release of infectious virus as compared to the human cell types. Additionally, both HPAIVs and LPAIVs belonging to various subtypes fail to spread efficiently among pigs, even when the nasal virus excretion levels approach those of SIVs (Shortridge et al. 1998, Loeffen et al. 2004, Choi et al. 2005, De Vleeschauwer et al. 2009b). Sustained pig-to-pig transmission of AIVs, which one would expect to be a minimum constraint to allow adjustment to a mammalian host, requires mutational adaptation to the new host. Such an event is illustrated by the avian-like H9N2 currently circulating in pigs in China. This virus, widespread in poultry, progressively gained mammalian host specificity by mutations in the HA (including L226) or by reassortment with HP H5N1 or H7N3 AIVs (Matrosovich et al. 2001, Fusaro et al. 2011, Yu et al. 2011). To date, the isolation of at least 10 different genotypes of H9N2 in Chinese pigs has been reported (Zhang et al. 2009) but still the virus is not maintained in the pig population.

Despite the Sia α2-6 binding preference of human influenza viruses, these viruses also appear not to be as replication competent in pigs as SIVs. A wholly human H3N2 A/Swine/Ontario/00130/97 for example, isolated from a pig in Canada, could not infect other pigs in the herd and was shown to have lower replication and shedding levels than a swine-adapted triple reassortment A/Swine/Minnesota/539/99 H3N2 under experimental conditions (Landolt et al. 2003). Additional *in vivo* (Landolt et al. 2006) and *in vitro* (Busch et al. 2008) experiments, using reverse genetics-generated reassortant viruses, demonstrated that these phenotypes are mainly dependent on the HA gene. A possible explanation could be the preferential binding of human influenza viruses to Neu5Ac rather than to Neu5Gc, while mass spectrometry analyses of pig tissues has shown that the latter is the most abundantly expressed Sia variant in this host (Higa et al. 1985, Suzuki et al. 1986, Varki 2001, Nicholls et al. submitted for publication).

When considering the experiment by Beare and Webster (1991) on the infection of human volunteers with AIVs, one has to keep in mind that an overinterpretation of these results might have taken place in the past. Most likely these individuals were not seronegative for influenza viruses just like most pigs in the field (Van Reeth et al. 2008). Therefore it is plausible that prior infections with human viruses were (at least partially) protecting these individuals from infection with avian viruses, falsely leading to the conclusion that humans are less susceptible to AIV infections than pigs. Evidence has been provided (Van Reeth et al. 2009, De Vleeschauwer and Van Reeth 2010) that prior infection of pigs with swine influenza viruses also partially protects them from infection with avian influenza viruses even when these belong to unrelated HA and NA subtypes.

The simultaneous expression of both α2-6 and α2-3 linked Sia in the same cells in porcine tissues may be less of an argument than previously hypothesized, particularly in the light of the mixing vessel theory. The first results by Ito et al. (1998b) and Suzuki et al. (2000) on the abundant expression of α2-3 linked Sia in the porcine trachea have been contradicted by several research groups. Applying lectin histochemistry, it was extensively demonstrated that α2-6 linked Sia receptors are dominant on the epithelial lining of trachea and bronchus whereas α2-3 linked Sia was hardly detected (Nelli et al. 2010, Van Poucke et al. 2010, Trebbien et al. 2011, Löndt et al. 2012). These findings are strengthened by the outcome of infection studies *in vivo* (Lipatov et al.2008, De Vleeschauwer et al. 2009a) and *ex vivo* (Van Poucke et al. 2010, Trebbien et al. 2011, Löndt et al. 2012) with avian influenza viruses as well as by their lack of attachment observed by Van Riel et al. (2007) using virus histochemistry. More extensive studies have additionally shown that the receptor expression pattern in pigs is not unique, but displays great similarities with humans, ferrets and domestic poultry (chickens, quails, turkeys) as shown in table 2. Given these findings, it is likely that some species of poultry and even humans could serve as a mixing vessel or as an intermediate host in the transmission of AIVs to land-based poultry and mammals as well (Thompson et al. 2006, Wan and Perez 2007, Hossain et al. 2008, Imai and Kawaoka 2012). Studying the alterations that resulted in the establishment of Asian duck H9N2 AIV in land-based poultry, Perez et al. (2003) found that quail are readily infected with non-adapted H9N2 AIVs while chickens were refractory to infection with these viruses. Changes in the HA-gene, associated with the adaptation to quail, rendered the virus transmissible to chickens as well. These authors, therefore, concluded that a virus adapted to quail is able to cross the species barrier a second time. In addition, most human cases of H5N1, H7N7 or H9N2 virus infection have occurred as a result of direct contact with sick or dead infected poultry (Claas et al. 1998, Koopmans et al. 2004, Gu et al. 2007, de Wit et al. 2010).

Studies on differences between the cell types targeted by avian (-like) and human (-like) influenza viruses in primary human respiratory epithelial cells have generated conflicting reports. When one replication cycle was studied in human pseudostratified mucociliairy epithelial cells, several researchers (Matrosovich et al. 2004b; 2007, Thompson et al. 2006, Wan and Perez 2007) concluded that viruses with an avian-like receptor binding profile prefered infection of ciliated cells while human-like influenza viruses prefered non-ciliated cells. Earlier research by Baum and Paulson (1990) and Couceiro et al. (1993), however, had revealed a difference in cell tropism that was exactly the opposite. Studying the attachment of labeled inactivated human and avian influenza viruses to different parts of the human upper respiratory tract, Van Riel et al. (2010) observed abundant and moderate attachment of human influenza viruses to ciliated and goblet cells respectively. In contrast, the AIV attached only rarely to ciliated epithelial cells and not to goblet cells. Gu et al. (2007), on the other hand, performed in situ hybridization combined with tubulin-β IHC to identify ciliated cells on tissues from 2 patients who had died from H5N1 AIV infection. These researchers found viral sequences in both ciliated and non-ciliated epithelial cells of the trachea.

Similar studies in porcine lung explants showed that a swine H3N2 (with α2-6 binding) and an avian H7N7 (with α2-3 binding) both were able to infect ciliated as well as goblet cells, although lectin staining had only identified α2-6 Sia expression on ciliated cells (Punyadarsaniya et al. 2011). Taken together, the exact implications of targeting different cell types on host range specificity and pathogenesis are not elucidated yet. Even the function of Sia as being the (only) internalizing receptor for influenza viruses is a point of discussion. Several authors suggested, based on different types of research, that Sias could provide an initial relatively low-affinity binding for influenza viruses that needs subsequent binding to another receptor (Kumari et al. 2007, Guo et al. 2009). Bateman et al. (2008) and Oshansky et al. (2011) demonstrated that replication of avian (-like) influenza viruses in swine respiratory epithelial cells and human bronchial epithelial cells respectively, can take place independent of α2-3 Sia expression. Glaser and coworkers (2007), on their turn, showed the same for human influenza viruses in knock-out mice lacking ST6 Gal I sialyltransferase. When Stray et al. (2000) enzymatically removed the surface Sias from MDCK-cells, infection with A/NWS/33 (H1N1) reassortants could only be reduced, not abolished. In a similar way, Thompson et al. (2006) assessed the remaining infectivity of A/England/26/99 (H3N2) leading to the same conclusions.

One of the arguments in favor of an important role of pigs in the human influenza ecology is the undeniable occurrence of reassortment events in this host all over the world (Castrucci et al. 1993, Claas et al. 1994, Shu et al. 1994, Zhou et al. 1999, Karasin et al. 2000b, Olsen 2002a, Olsen et al.

2004, Zell et al. 2008, Fan et al. 2012, Starick et al. 2012), involving human, avian and swine influenza virus genes. The processes underlying reassortment however are not fully understood. A key requirement for reassortment to occur, is the co-infection of the same cell by 2 viruses with different HA types. Both *in vitro* and *in vivo* studies have shown that the NA expressed on virion producing cells is removing both α2-3 and α2-6 linked Sias (also from surrounding non-infected cells), raising the question how a second influenza virus is able to infect the same cell (Huang et al. 2008, Trebbien et al. 2011, Nicholls personal communications). Kida et al. (1994) even demonstrated the rescue of a reassortant virus containing avian genes derived from an avian virus that was not capable of replicating in pigs.

The similarity between the HA subtypes endemically circulating in pigs and humans, being H1, H3 and H1, H2 and H3 respectively strengthens the relation between both hosts in influenza ecology as does the fast turn-over of pigs resulting in the permanent presence of susceptible seronegative animals (Ma et al. 2009). After large scale glycan microarray analysis studies of classical swine and human seasonal H1N1 influenza viruses, Chen et al. (2011) stated that "a conserved specificity for similar α2-6-sialoside receptors was maintained in spite of a long term circulation in separate hosts, suggesting that humans and pigs impose analogous selection pressures on the evolution of receptor binding function." The recently reported infections of humans in the U.S.A. (296 confirmed cases to date) with an H3N2 after direct exposure to pigs emphasize the interplay between both hosts. This virus was identified as a swine TR H3N2 that acquired the M-gene from pH1N1 and seems more easily transmissible to humans than other SIVs.

Since Scull et al. (2009) and Zeng et al. (2013) provided evidence that viruses with an avian or avian-like surface glycoprotein have a restricted replication potential in human airway epithelium at temperatures of 32-33°C (representative for the upper respiratory tract temperature *in vivo*) but not at 37°C or higher (matching lower respiratory tract temperatures), another motivation to support the intermediate role of pigs came up. The normal body temperature of adult pigs is around 38,5°C while that of piglets is around 39 to 39,5°C. This is in between the normal average body temperatures of humans (37°C) and birds (40°C).

1.6. References

Alexander D.J. (2000). A review of avian influenza in different bird species. Vet Microbiol 74: 3-13.

Anthony S.J., St. Leger J.A., Pugliares K. et al. (2012). Emergence of Fatal Avian Influenza in New England Harbor Seals. mBio 3(4):e00166-12.doi:10.1128/m.Bio e00166-12.

Banks J., Speidel E.S., Moore E. et al. (2001). Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol 2001: 963-973.

Bansil R. and Turner B.S. (2006). Mucine structure, aggregation, physiological functions and biomedical applications. Current Opinion in Colloid & Interface Science 11: 164-170.

Barbé F., Atanasova K., Van Reeth K. (2011). Cytokines and acute phase proteins during the acute stage of a swine influenza virus infection. Vet J 187: 48-53.

Bateman A.C., Busch M.G., Karasin D.A. et al. (2008). Amino Acid 226 in the Hemagglutinin of H4N6 Influenza Virus Determines Binding Affinity for α2,6-Linked Sialic Acid and Infectivity Levels in Primary Swine and Human Respiratory Epithelial Cells. J Virol 82: 8204-8209.

Bateman A.C., Karamanska R., Busch M.G. et al. (2010). Glycan Analysis and Influenza A Virus Infection of Primary Swine Respiratory Epithelial Cells. J Biol Chem 285: 34016-34026.

Baum L.G. and Paulson J.C. (1990). Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. Acta Histochem Suppl 40: 35-38.

Bean W.J., Schell M., Katz J. et al. (1992). Evolution of the H3 Influenza Virus Hemagglutinin from Human and Nonhuman Hosts. J Virol 66: 1129-1138.

Beare A.S. and Webster R.G. (1991). Replication of avian influenza viruses in humans. Arch Virol 119: 37-42.

Bridges C.B., Lim W., Hu-Primmer J. et al. (2002). Risk of influenza A (H5N1) infection among poultry workers, Hong Kong, 1997-1998. J Inf Dis 185: 1005-1010.

Brown I.H., Hill M.L., Harris P.A. et al. (1997a). Genetic characterisation of an influenza A virus of unusual subtype (H1N7) isolated from pigs in England. Arch Virol 142: 1045-1050.

Brown I.H., Ludwig S., Olsen C.W. et al. (1997b). Antigenic and genetic analyses of H1N1 influenza A viruses from European pigs. J Gen Virol 78: 553-562.

Brown I.H., Harris P.A., McCauley J.W. et al. (1998). Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. J Gen Virol 79: 2947-2955.

Brown I.H. (2000). The epidemiology and evolution of influenza viruses in pigs. Vet Microbiol 74: 29-46.

Busch M.G., Bateman A.C., Landolt G.A. et al. (2008). Identification of amino acids in the HA of H3 influenza viruses that determine infectivity levels in primary swine respiratory epithelial cells. Virus Res 133: 269-279.

Campitelli L., Donatelli I., Foni E. et al. (1997). Continued Evolution of H1N1 and H3N2 Influenza Viruses in Pigs in Italy. Virology 232: 310-318.

Capua I., Marangon S., Selli L. et al. (1999). Outbreaks of highly pathogenic avian influenza (H5N2) in Italy during October 1997 to January 1998. Avian Pathol 28: 455-460.

Castrucci M.R., Donatelli I., Sidoli L. et al. (1993). Genetic Reassortment between Avian and Human Influenza A viruses in Italian Pigs. Virology 193: 503-506.

Chandrasekaran A., Srinivasan A., Raman R. et al. (2008). Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. Nat Biotech 26: 107-113.

Chen W., Calvo P. A., Malide D. et al. (2001). A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7: 1306-1312.

Chen L., Rivailler P., Hossain J. et al. (2011). Receptor specificity of subtype H1 influenza A viruses isolated from swine and humans in the United States. Virology 412: 401-410.

Choi Y.K., Lee J.H., Erickson G. et al. (2004). H3N2 Influenza Virus Transmission from Swine to Turkeys, United States. Em Inf Dis 10: 2156-2160.

Choi Y.K., Nguyen T.D., Ozaki H. et al. (2005). Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol 79: 10821-10825.

Chutinimitkul S., van Riel D., Munster V.J. et al. (2010). In vitro assessment of attachment pattern and replication efficiency of H5N1 influenza A viruses with altered receptor specificity. J Virol 84: 6825-6833.

Chu V.C. and Whittaker G.R. (2004). Infuenza virus entry and infection require host cell N-linked glycoprotein. PNAS 101: 18153-18158.

Claas E.C.J., Kawaoka Y., de Jong J. et al. (1994). Infection of Children with Avian-Human Reassortant Influenza Virus from Pigs in Europe. Virology 204: 453-457.

Claas E.C.J., Osterhaus A.D.M.E., van Beek R. et al. (1998). Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. The Lancet 351: 472-477.

Colman P.M. (1994). Influenza virus neuraminidase: Structure, antibodies, and inhibitors. Protein Science 3: 1687-1696.

Conenello G. M. and Palese P. (2007). Influenza A virus PB1-F2: a small protein with a big punch. Cell Host Microbe 2: 207-209.

Conenello G.M., Zamarin D., Perrone L.A. et al. (2007). A Single Mutation in the PB1-F2 of H5N1 (HK/97) and 1918 Influenza A Virus Contributes to Increased Virulence. PLoS Pathogens 3: 1414-1421.

Connor R.J., Kawaoka Y., Webster R.G. et al. (1994). Receptor Specificty in Human, Avian, and Equine H2 and H3 Influenza Virus Isolates. Virology 205: 17-23.

Couceiro J.N.S.S., Paulson J.C., Baum L.G. (1993). Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res 29: 155-165.

Couceiro J.N.S.S. and Baum L.G. (1994). Characterization of the Hemagglutinin Receptor Specificity and Neuraminidase Substrate Specificity of Clinical Isolates of Human Influenza A Viruses. Mem Inst Oswaldo Cruz 89: 587-591.

Cross K.J., Langley W.A., Russell R.J. et al. (2009). Composition and Functions of the Influenza Fusion Peptide. Protein & Peptide Letters 16: 766-778.

Daniels R.S., Jeffries S.J., Yates P. et al. (1987). The receptor-binding and membrane-fusion properties of influenza virus variants selected using anti-haemagglutinin monolconal antibodies. EMBO Journal 6: 1459-1465.

Daoust P., Kibenge F.S.B., Fouchier R.A.M. et al. (2011). Replication of low pathogenic avian influenza virus in naturally infected mallard ducks (*Anas Platyrhynchos*) causes no morphologic lesions. J Wildlife Dis 47: 401-409.

Das K., Ma L., Xiao R. et al. (2008). Structural basis for the suppression of a host antiviral response by influenza A virus. PNAS 105: 13092-13097.

Das K., Aramini J.M., Ma L. et al. (2010). Structures of influenza A proteins and insights into antiviral drug targets. Nat Struct Molec Biology 17: 530-538.

de Jong M.D., Qui P.T., Hien V.M. et al. (2005). Fatal Avian Influenza A (H5N1) in a Child Presenting with Diarrhea Followed by Coma. N Engl J Med 352: 686-691.

de Jong M.D., Simmons C.P., Tan Thahn T. et al. (2006). Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med 12: 1203-1207.

de Jong J.C., Smith D.J., Lapedes A.S. et al. (2007). Antigenic and Genetic Evolution of Swine Influenza A (H3N2) Viruses in Europe. J Virol 81: 4315-4322.

De Vleeschauwer A., Atanasova K., Van Borm S., et al. (2009a). Comparative Pathogenesis of an Avian H5N2 and a Swine H1N1 Influenza Virus in Pigs. PLoS ONE 4 doi:10.1371/journal.pone.0006662.

De Vleeschauwer A., Van Poucke S., Braeckmans D., et al. (2009b). Efficient transmission of swine-adapted but not wholly avian influenza viruses between pigs and from pigs to ferrets. J Infect Dis 200:1884-1892.

De Vleeschauwer A. and Van Reeth K. (2010). Prior infection of pigs with swine influenza viruses is a barrier to infection with avian influenza viruses. Vet Microbiol 146: 340-345.

De Vleeschauwer A., Van Poucke S., Karasin A.I. et al. (2011). Cross-protection between antigenically distinct H1N1 swine influenza viruses from Europe and North America. Influenza Other Resp. Viruses 5: 115-122.

de Wit E., Munster V.J., van Riel D. et al. (2010). Molecular Determinants of Adaptation of Highly Pathogenic Avian Influenza H7N7 Viruses to efficient Replication in the Human Host. J Virol 84: 1597-1606.

Dias A., Bouvier D., Crépin T. et al. (2009). The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458: 914-918.

Domingo E. and Holland J.J. (1997). RNA virus mutations and fitness for survival. Annu Rev Microbiol 51: 151-178.

Dugan V.G., Chen R., Spiro D.J. et al. (2008). The Evolutionary Genetics and Emergence of Avian Influenza Viruses in Wild Birds. PLoS Pathogens 4: doi:10.1371/journal.ppat.1000076.

Dunham E.J., Dugan V.G., Kaser E.K. et al. (2009). Different Evolutionary Trajectories of European Avian-Like and Classical Swine H1N1 Influenza A Viruses. J Virol 83: 5485-5494.

Elbers A.R.W., Koch G., Bouma A. (2005). Performance of clinical signs in poultry for the detection of outbreaks during the avian influenza A (H7N7) epidemic in the Netherlands. Avian Pathol 34: 181-187.

Fan X., Zhu H., Zhou B. et al. (2012). Emergence and Dissemination of a Swine H3N2 Reassortant Influenza Virus with 2009 Pandemic H1N1 Genes in Pigs in China. J Virol 86: 2375-2378.

Fiers W., De Filette M., El Bakkouri K. et al. (2009). M2e-based universal influenza A vaccine. Vaccine 45: 6280-6283.

Fouchier R.A.M., Osterhaus A.D.M.E., Brown I.A. (2003). Animal influenza surveillance. Vaccine 21: 1754-1757.

Franciosi C., D'Aprile P.N., Alexander D.J et al. (1981). Infuenza A virus infections in commercial turkeys in North East Italy. Avian Pathol 10: 303-311.

Franck N., Queguiner S., Gorin S. et al. (2007). Molecular epidemiology of swine influenza virus in France: identifications of novel H1N1 reassortants. In: Markowska-Daniel, I. (ed.), 5th International Symposium on Emerging and Re-emerging Pig Diseases, PHV Kopiorama (K. Karez, G. Kozlowski), Krakow, Poland, 24–27 June, p. 250.

Fusaro A., Monne I., Salviato A. et al. (2011). Phylogeography and Evolutionary History of Reassortment H9N2 Viruses with Potential Human Health Implications. J Virol 85: 8413-8421.

Gabriel G., Dauber B., Wolff T. et al. (2005). The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. PNAS 102: 18590-18595.

Gabriel G., Herwig A., Klenk H. (2008). Interaction of Polymerase Subunit PB2 and NP with Importin α1 Is a Determinant of Host Range of Influenza A Virus. PLoS Pathogens 4: doi:10.1371/journal.ppat.0040011.

Gambaryan A.S. and Matrosovich M.N. (1992). A solid-phase enzyme linked assay for influenza virus receptor-binding activity. J Virol Methods 39: 111-123.

Gambaryan A.S., Tuzikov A.B., Piskarev V.E. et al. (1997). Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl(N-acetyllactosamine). Virology 232: 345-350.

Gambaryan A., Yamnikova S, Lvov D. et al. (2005). Receptor specificity of influenza viruses from birds and mammals: new data on involvement of the inner fragments of the carbohydrate chain. Virology 334: 276-283.

Gamblin S.J. and Skehel J.J. (2010). Influenza Hemagglutinin and Neuraminidase Membrane Glycoproteins. J Biol Chem 285: 28403-28409.

Gaydos J.C., Top F.H., Hodder R.A. et al. (2008). Swine influenza A outbreak, Fort Dix, New Jersey, 1976. Em Inf Dis 12: 23-28.

Gibbs A.J., Armstrong J.S., Downie J.C. (2009). From where did the 2009 'swine-origin' influenza A virus (H1N1) emerge? Virol J 6: doi:10.1186/1743-422X-6-207.

Gillim-Ross L. and Subbarao K. (2007). Can Immunity Induced by the Human Influenza Virus N1 Neuraminidase Provide Some Protection from Avian Influenza H5N1 Viruses? PLoS Medicine 4: doi:10.1371/journal.pmed.0040091.

Glaser L., Stevens J., Zamarin D. et al. (2005). A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. J Virol 79: 11533-11536.

Glaser L., Conenello G., Paulson J. et al. (2007). Effective replicatin of human influenza viruses in mice lacking a major α2,6 sialyltransferase. Virus Res 126: 9-18.

Gong J., Xu W., Zhang J. (2007). Structure and functions of influenza virus neuraminidase. Current Med Chem 14: 113-122.

Gorman O.T., Bean W.J., Kawaoka Y. et al. (1991). Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses. J Virol 65: 3704-3714.

Gray G.C., McCarthy T., Capuano A.W. et al. (2007). Swine Workers and Swine Influenza Virus Infections. Em Inf Dis 13: 1871-1878.

Guan Y., Shortridge K.F., Krauss S. et al. (1996). Emergence of Avian H1N1 Influenza Viruses in Pigs in China. J Virol 70: 8041-8046.

Gu Y., Xie Z., Gao Z. et al. (2007). H5N1 infection of the respiratory tract and beyond: a molecular pathology study.The Lancet 370: 1137-1145.

Guo C., Takahashi N., Yagi H. et al. (2007). The quail and chicken intestine have sialyl-galactose sugar chains responsible for the binding of influenza A viruses to human type receptors. Glycobiology 17: 713-724.

Guo Y., Rumschlag-Booms E., Wang J. et al. (2009). Analysis of hemaggutinin-mediated entry tropism of H5N1 avian influenza. Virol J 6: doi: 10.1186/1743-422X-6-39.

Haesebrouck F. and Pensaert M. (1988). Influenza in swine in Belgium (1969-1986): epizootiologic aspects. Comp Immunol Microbiol Infect Dis 11: 215-222.

Hale B.G., Randall R.E., Ortin J. et al. (2008). The multifunctional NS1 protein of influenza A viruses. J Gen Virol 89: 2359-2376.

Harris A., Cardone G., Winkler D.C. et al. (2006). Influenza virus pleiomorphy characterized by cryoelectron tomography. PNAS 103: 19123-19127.

Heinen P.P., de Boer-Luijtze E., Bianchi A.T.J. (2001). Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection. J Gen Virol 82: 2697-2707.

Hensley S.E., Das S.R., Bailey A.L. et al. (2009). Hemagglutinin Receptor Binding Avidity Drives Influenza A Virus Antigenic Drift. Science 326: doi:10.1123/science.1178258.

Herfst S., Chutinimitkul S., Ye J. et al. (2010). Introduction of Virulence Markers in PB2 of Pandemic Swine-Origin Influenza Virus Does Not Result in Enhanced Virulence or Transmission. J Virol 84: 3752-3758.

Herfst S., Schrauwen E.J.A., Linster M. et al. (2012). Airborne Transmission of Influenza A/H5N1 Virus Between Ferrets. Science 336: 1534-1541.

Hidari K.I.P.J. and Suzuki T. (2010). Glycan Receptor for Influenza Virus. The Open Antimicrobial Agents Journal 2: 26-33.

Higa H.H., Rogers G.N., Paulson J.J. (1985). Influenza Virus Hemagglutinins Differentiate between Receptor Determinants Bearing N-Acetyl-, N-Glycollyl-, and N,O-Diacetylneuraminic Acids. Virology 144: 279-282.

Hillaire M.L.B., van Eijk M., van Trierum S.E. et al. (2011a). Assessment of the Antiviral Properties of Recombinant Porcine SP-D against Various Influenza A Viruses In Vitro. PLoS ONE 6: doi:10.1371/journal.pone.0025005.

Hillaire M.L.B., van Trierum S.E., Kreijz J.H.C.M. et al. (2011b). Cross-protective immunity against influenza pH1N1 2009 viruses induced by seasonal influenza A (H3N2) virus is mediated by virus-specific T-cells. J Gen Virol 92: 2339-2349.

Hjulsager C.K., Bragstad K., Botne A. et al. (2006). Isolation and genetic characterization of new reassortant H1N2 swine influenza virus from pigs in Denmark. In:Leitao A., and C. Martins (eds), 7th International Congress of Veterinary Society, Lisboa, Portugal, p. 133.

Hofshagen M., Gjerset B., Er C. et al. (2009). Pandemic influenza A (H1N1)v: human to pig transmission in Norway? Euro Surveill 14: pii19406.

Hossain M.J., Hickman D., Perez D.R. (2008). Evidence of expanded host range and mammalian-associated genetic changes in a duck H9N2 influenza virus following adaptation in quail and chickens. PLoS ONE 3: doi: 10.1371/journal.pone.0003170

Huang I., Li W., Sui J. et al. (2008). Influenza A Virus Neuraminidase Limits Viral Superinfection. J Virol 82: 4834-4843.

Hulse D.J., Webster R.G., Russel R.J. et al. (2004). Molecular Determinants within the Surface Proteins Involved in the Pathogenicity of H5N1 Influenza Viruses in Chickens. J Virol 78: 9954-9964.

Imai M. and Kawaoka Y. (2012). The role of receptor binding specificity in interspecies transmission of influenza viruses. Current Opinion in Virology 2: 160-167.

Imai M., Watanabe T., Hatta M. et al. (2012). Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature doi:10.1038/nature10831

Ito T., Suzuki Y., Mitnaul L. et al. (1997). Receptor Specificity of Influenza A Viruses Correlates with the Agglutination of Erythrocytes from Different Animal Species. Virology 227: 493-499.

Ito T. and Kawaoka Y. (1998a). Avian Influenza. In: Nicholson K.G., Webster R.G., Hay A.J. (Eds) Textbook of Influenza. Blackwell Science Ltd., Malden, MA, USA, p. 126-136.

Ito T., Couceiro J.N.S.S., Kelm S. et al. (1998b). Molecular Basis for the Generation in Pigs of Influenza A Viruses with Pandemic Potential. J Virol 72: 7367-7373.

Jongkon N., Mokmak W., Chuakheaw D. et al. (2009). Prediction of avian influenza A binding preference to human receptor using conformational analysis of receptor bound to hemagglutinin. BMC Genomics 10:S24.

Joshi S.R., Shaw A.C, Quagliarello V.J. (2009). Pandemic Influenza H1N1 2009, Innate Immunity, and the Impact of Immunosenescence on Influenza Vaccine. Yale Journal of Biology and Medicine 82: 143-151.

Jung T., Choi C., Chae C. (2002). Localization of Swine Influenza Virus in Naturally Infected Pigs. Vet Pathol 39: 10-16.

Karasin A.I., Brown I.H., Carman S. et al. (2000a). Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. J Virol 74: 9322-9327.

Karasin A.I., Schutter M.M., Cooper L.A. et al. (2000b). Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977-1999: Evidence for wholly human and reassortant virus genotypes. Virus Res 68: 71-85.

Karasin A.I., West K., Carman S. et al. (2004). Characterization of Avian H3N3 and H1N1 Influenza A Viruses Isolated from Pigs in Canada. J Clin Microbiol 42: 4349-4354.

Katz J.M. (2003). The impact of avian influenza viruses on public health. Avian Dis 47: 914-920.

Kawai T. and Akira S. (2009). The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immun 21: 317-337.

Kida H., Ito T., Yasuda J. et al. (1994). Potential for transmission of avian influenza viruses to pigs. J Gen Virol 75: 2183-2188.

Kim C., Lee K., Han K. et al. (2011). Expression of mucins on the mucosal surface of lungs of 4 week-old pigs. Vet Res Commun 35: 255-260.

Kimble B., Nieto G.R., Perez D.R. (2010). Characterization of influenza virus sialic acid receptors in minor poultry species. Virol J 7: 365.

Kim W.I., Wu W.H., Janke B. et al. (2006). Characterization of the humoral immune response of experimentally infected and vaccinated pigs to swine influenza viral proteins. Arch Virol 151: 23-36.

Kirkeby S., Martel C.J.M., Aasted B. (2009). Infection with human H1N1 influenza virus affects the expression of sialic acids of metaplastic mucous cells in the ferret airways. Virus Res 144: 225-232.

Kishida N., Sakoda Y., Eto M. et al. (2004). Co-infection of Staphylococcus aureu or Haemophilus paragallinaru exacerbates H9N2 influenza A virus infection in chickens. Arch Virol 149: 2095-2104.

Kitikoon P., Sreta D., Ayudhya S.N. et al. (2011). Brief report: Molecular characterization of a novel reassorted pandemic H1N1 2009 in Thai pigs. Virus Genes 43: 1-5.

Kobasa D., Kodihalli S., Luo M. et al. (1999). Amino Acid Residues Contributing to the Substrate Specificty of the Influenza A Virus Neuraminidase. J Virol 73: 6743-6751.

Koopmans M., Wilbrink B., Conyn M. et al. (2004). Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. The Lancet 363: 587-593.

Korteweg C. and Gu J. (2008). Pathology, Molecular Biology, and Pathogenesis of Avian Influenza A (H5N1) Infection in Humans. AJP 172: 1155-1170.

Kuchipudi S.V., Nelli R., Whie G.A. et al. (2009). Differences in influenza virus receptors in chickens and ducks: Implications for interspecies transmission. J Mol Genet Med 3: 143-151.

Kuiken T., Holmes E.C., McCauley J. et al. (2006). Host Species Barriers to Influenza Virus Infections. Science 312: 394-397.

Kumari K., Gulati S., Smith D.F. et al. (2007). Receptor binding specificity of recent human H3N2 influenza viruses. Virology J 4: doi:10.1186/1743-422X-4-42.

Kuntz-Simon G. and Madec F. (2009). Genetic and Antigenic Evolution of Swine Influenza Virusess in Europe and Evaluation of Their Zoonotic Potential. Zoonose and Public Health 56: 310-325.

Lakadamyali M., Rust M. J., Zhuang X. (2004). Endocytosis of influenza viruses. Microbes and Infection 6: 929-936.

Lamb R.A. and Krug R.M. (2001). Orthomyxoviridae: the viruses and their replication. In: Knipe D.M., Howley P.M. (Eds.) Fields Virology, Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, USA, p. 1487-1531.

Landolt G.A., Karasin A.I., Phillips L. et al. (2003). Comparison of the Pathogenesis of Two Genetically Different H3N2 Influenza A Viruses in Pigs. J Clin Microbiol 41: 1936-1941.

Landolt G.A., Karasin A.I., Schutten M.M. et al. (2006). Restricted infectivity of a human-lineage H3N2 influenza A virus in pigs is hemagglutinin and neuraminidase gene dependent. J Clin Microbiol 44: 297-301.

Larsen D.L., Karasin A., Zuckermann F. et al. (2000). Systemic and mucosal immune responses to H1N1 influenza virus infection in pigs. Vet Microbiol 74: 117-131.

Lekcharoensuk P., Lager K.M., Vemulapalli R. et al. (2006). Novel Swine Influenza Virus Subtype H3N1, Unites States. Em Inf Dis 12: 787-793.

Liao H., Hsu C., Wang S. et al. (2010). Differential Receptor Binding Affinities of Influneza Hemagglutinins on Glycan Arrays. J Am Chem Soc 132: 14849-14856.

Li J., zu Dohna H., Cardona C.J. et al. (2011). Emergence and Genetic Variation of Neuraminidase Stalk Deletions in Avian Influenza Viruses. PLoS ONE 6: doi:10.1371/journal.pone.0014722.

Lipatov A.S., Kwon Y.K., Sarmento L.V., et al. (2008). Domestic pigs have low susceptibility to H5N1 highly pathogenic avian influenza viruses. PloS Pathog 4.doi: 10.1371/journal.ppat.1000102.

Liu J., Xiao H., Lei F. et al. (2005). Highly Pathogenic H5N1 Influenza Virus Infection in Migratory Birds. Science 309: 1206.

Liu Q., Ma J., Liu, H.et al. (2012). Emergence of novel reassortant H3N2 swine influenza viruses with the 2009 pandemic H1N1 genes in the United States. Arch of Virol 157: 555-562.

Loeffen W., de Boer E., Koch G. (2004). Transmission of a highly pathogenic avian influenza virus to swine in the Netherlands. In: Program and abstracts of the International Society for Animal Hygiene. Saint Malo, France, 11-13 October, 329-330.

Löndt B. Z., Brookes S.M., Nash B.J. et al. (2012). The infectivity of pandemic 2009 H1N1 and avian influenza viruses for pigs: an assessment by *ex vivo* respiratory tract organ culture. Influenza and other Resp Viruses doi: 10.1111/j.1750-2659.2012.00397.x

Maines T.R., Szretter K.J., Perrone L. et al. (2008). Pathogenesis of emerging avian influenza viruses in mammals and the host innate immune response. Immun Rev 225: 68-84.

Maldonado J., Van Reeth K., Riera P. et al. (2006). Evidence of the concurrent circulation of H1N2, H1N1 and H3N2 influenza A viruses in densely populated pig areas in Spain. Vet J 172: 377-381.

Marozin S., Gregory V., Cameron K. et al. (2002). Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. J GenVirol 83: 735-745.

Massin P., van der Werf S., Naffakh N. (2011). Resdiue 627 of PB2 Is a Determinant of Cold Sensitivity in RNA Replication of Avian Influenza Viruses. J Virol 75: 5398-5404.

Matrosovich M.N., Gambaryan A.S., Teneberg S. et al. (1997). Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosed and by a higher conservation of the HA Receptor-Binding Site. Virol 233: 224-234.

Matrosovich M., Tuzikov A., Bovin N. et al. (2000). Early Alterations of the Receptor-Binding Properties of H1, H2, and H3 Avian Influenza Virus Hemagglutinins after Their Introduction into Mammals. J Virol 74: 8502-8512.

Matrosovich M.N., Krauss S., Webster R.G. (2001). H9N2 Influenza A Viruses from Poultry in Asia Have Human Virus-like Receptor Specificity. Virology 281: 156-162.

Matrosovich M. and Klenk H. (2003). Natural and synthetic sialic acid-containing inhibitors of influenza virus receptor binding. Rev Med Virol 13: 85-97.

Matrosovich M.N., Matrosovich T.Y., Gray T. et al. (2004a). Neuraminidase Is Important for the Initiation of Influenza Virus Infection in Human Airway Epithelium. J Virol 78: 12665-12667.

Matrosovich M.N., Matrosovich T.Y., Gray T. et al. (2004b). Human and avian influenza viruses target differenct cell types in cultures of human airway epithelium. PNAS 101: 4620-4624.

Matrosovich M.N., Klenk H., Kawaoka Y. (2006). Receptor specificity, host-range, and pathogenicity of influenza viruses. In: Kawaoka Y. (Ed.) Influenza virology: current topics. Caister Academic Press, Wymondham, Norfolk, England, p. 95-137.

Matrosovich M., Matrosovich T., Uhlendorff J. et al. (2007). Avian-virus-like specificity of the hemagglutinin impedes influenza virus replication in cultures of human airway epithelium. Virology 361: 384-390.

Matrosovich M.N., Gambaryan A.S., Klenk H. (2008). Receptor Specificity of Influenza Viruses and Its alteration during Interspecies Transmission. In: Klenk H., Matrosovich M.N., Stech J. (Eds) Avian Influenza.

Matrosovich M., Stech J., Klenk H. (2009). Influenza receptors, polymerase and host range. Rev sci tech Off int Epiz 28: 203-217.

Ma W., Lager K.M., Vincent A.L. et al. (2009). The Role of Swine in the Generation of Novel Influenza Viruses. Zoonoses and Public Health 56: 326-337.

Meng B., Marriott A.C., Dimmock N.J. (2010). The receptor preference of influenza viruses. Influenza Other Resp Viruses 4: 147-153.

Min J. and Krug R. M. (2006). The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2 '-5 ' oligo (A) synthetase/RNase L pathway. PNAS 103: 7100-7105.

Mochalova L., Kurova V., Shtyrya Y. et al. (2007). Oligosaccharide specificity of influenza H1N1 virus neuraminidases. Arch Virol 152: 2047-2057.

Morales A.C.J., Hilt D.A., Williams S.M. et al. (2009). Biological characterisation of H4, H6 and H9 type low pathogenicity avian influenza viruses from wild birds in chickens and turykeys. Avian Dis 53: 552-562.

Mutinelli F., Capua I., Terregino C., Cattoli G. (2003). Clinical, Gross and Microscopic Findings in Different Avian Species Naturally Infeced During the H7N1 Low- and High Pathogenicity Avian Influenza Epidemics in Italy During 1999-2000. Avian Dis 47: 844-848.

Myers K.P., Olsen C.W., Gray G.C. (2007). Cases of Swine Influenza in Humans: A Review of the Literature. Clin Infect Dis 44: 1084-1088.

Nayak D.P., Balogun R.A., Yamada H et al. (2009). Influenza virus morphogenesis and budding. Virus Res 143: 147-161.

Nelli R.K., Kuchipudi S.V., White G.A. et al. (2010) Comparative distribution of human and avian type sialic acid influenza receptors in the pig. Vet Res 6: doi:10.1186/1746-6148-6-4.

Nelli R.K., Dunham S.P., Kuchipudi S.V. et al. (2012). Mammalian Innate Resistance to Highly Pathogenic Avian Influenza H5N1 Virus Infection is Mediated through Reduced Pro-inflammation and Infectious Virus Release. J Virol: doi:10.1128/JVI.00244-12.

Neumann G., Hughes M. T., Kawaoka Y. (2000). Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. EMBO Journal 19: 6751-6758.

Neumann G. and Kawaoka Y. (2006). Host Range Restriction and Pathogenicity in the Context of Influenza Pandemic. Em Inf Dis 12: 881-886.

Newhouse I.E., Xu D., Markwick P.R.L. et al. (2009). Mechanism of Glycan Receptor Recognition and Specificty Switch for Avian, Swine, and Human Adapted Influenza Virus Hemagglutinins: A Molecular Dynamics Perspective. J Am Chem Soc 131: 309-320.

Nicholls J.M., Bourne A.J., Chen H. et al. (2007a). Sialic acid receptor detection in the human respiratory tract: evidence for widespread distribution of potential binding sites for human and avian influenza viruses. Resp Res 8: doi:10.1186/1465-9921-8-73.

Nicholls J.M., Chan M.C., Chan W.Y. (2007b). Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nat Med 13: 147-149.

Nicholls J.M., Chan R.W.Y., Russell R. et al. (2008). Evolving complexities of influenza virus and its receptors. Trends in Microbiol 16: 149-157.

Nidom C.A., Takano R., Yamada S. et al. (2010). Influenza A (H5N1) viruses from pigs, Indonesia. Emerg Infect Dis doi: 10.3201/eid1610.100508

Ohuchi M., Ohuchi R., Feldmann A. et al. (1997). Regulation of Receptor Binding Affinity of Influenza Virus Hemagglutinin by its Carbohydrate Moiety. J Virol 71: 8377-8384.

Olsen C.W. (2002a). The emergence of novel swine influenza viruses in North America. Virus Res 85: 199-210.

Olsen C.W., Brammer L., Easterday B.C. et al. (2002b). Serologic evidence of H1 swine influenza virus infection in swine farm residents and employees. Em Inf Dis 8: 814-819.

Olsen C.W., Landolt G.A., Karasin A.I. (2004). The emergence of novel influenza viruses among pigs in North America due to interspecies transmission and reassortment. International Congress Series 1236: 196-199.

Olsen B., Munster V.J., Wallensten A. et al. (2006a). Global Patterns of Influenza A Virus in Wild Birds. Science 312: 384-388.

O'Neill R.E., Talon J., Palese P. (1998). The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. EMBO Journal 17: 288-296.

Oshansky C.M., Pickens J.A., Bradley K.C. et al. (2011). Avian Inlfuenza Viruses Infect Primary Human Bronchial Epithelial Cells Unconstrained by Sialic Acid α2,3 Residues. PLoS ONE 6: doi: 10.1371/journal.pone.0021183.

Paulson J.C. and Rogers G.N. (1987). Resialylated erythrocytes for assessment of the specificity of sialyloligosaccharide binding proteins. Meth Enzymol 138: 162-168.

Payungporn S., Crawford C.P., Kouo T.S. et al. (2008). Influenza A Virus (H3N8) in Dogs with Respiratory Disease, Florida. Em Inf Dis 14: 902-908.

Peiris M., Yuen K.Y., Leung C.W. et al. (1999). Human infection with influenza H9N2. The Lancet 354: 916-917.

Peiris M.J.S., Yu W.C., Leung C.W. et al. (2004). Re-emergence of fatal human influenza A subtype H5N1 disease. The Lancet 363: 617-619.

Peiris M.J.S. (2009a). Avian influenza viruses in humans. Rev Sci tech Off int Epiz 28: 161-174.

Peiris M.J.S., Cheung C.Y., Leung C.Y.H. et al. (2009b). Innate immune responses to influenza A H5N1: friend or foe? Trends in Immun 30: 574-584.

Pensaert M., Ottis K., Vandeputte J. et al. (1981). Evidence for the natural transmission of influenza A virus from wild ducks to swine and its potential importance for man. Bulletin of the WHO 59: 75-78.

Perez D.R., Wilina L., Seiler J.P. et al. (2003). Role of Quail in the Interspecies Transmission of H9 Influenza A Viruses: Molecular Changes on HA That Correspons to Adaptation from Ducks to Chickens. J Virol 77: 3148-3156.

Perkins L.E., Swayne D.E. (2001). Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. Vet Pathol 38: 149-164.

Pillai S.P.S. and Lee C.W. (2010). Species and age related differences in the type and distribution of influenza virus receptors in different tissues of chickens, ducks and turkeys. Virol J 7: doi:10.1186/1743-422X-7-365

Portela A. and Digard P. (2002). The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. J Gen Virol 83: 723-734.

Post J., Burt D.W., Cornelissen J.B.W.J. et al. (2012). Systemic virus distribution and host responses in brain and intestine of chickens infected with low pathogenic or high pathogenic avian influenza viurs. Vir J 9: doi:10.1186/1743-422X-9-61.

Punyadarsaniya D., Liang C., Winter. et al. (2011). Infection of Differentiated Porcine Airway Epithelial Cells by Influenza Virus: Differential Susceptiblity to Infection by Porcine and Avian Viruses. PloS ONE 6: doi:10.1371/journal.pone.0028429

Ramos I., Bernal-Rubio D., Dunham N. et al. (2011). Effects of Receptor Binding Specificity of Avian Influenza Virus on the Human Innate Immune Response. J Virol 85: 4421-4431.

Roberts K.L., Shelton H., Scull M. et al. (2011). Lack of transmission of a human influenza virus with avian receptor specificity between ferrets is not due to decreased virus shedding, but rather a lower infectivity in vivo. J Gen Virol 92: 1822-1831.

Ruigrok R.W.H. (1998). Structure of Influenza A, B and C Viruses. In: Nicholson K.G., Webster R.G., Hay A.J. (Eds) Textbook of Influenza. Blackwell Science Ltd., Malden, MA, USA, p. 29-42.

Sawada T., Fedorov D.G., Kitaura K. (2010). Role of the Key Mutation in the Selective Binding of Avian and Human Influenza Hemagglutinin to Sialosides Revealed by Quantum-Mechanical Calculations. J Am Chem Soc 132: 16862-16872.

Scharfman A., Lamblin G., Roussel P. (1995). Interactions between human respiratory mucins and pahtogens. Bioch Soc Transactions 23: 836-839.

Scholtissek C., Bürger H., Bachmann P.A. et al. (1983). Genetic relatedness of hemagglutinins of the H1 subtype of influenza A viruses isolated from swine and birds. Virology 129: 521-523.

Scholtissek C., Bürger H., Kistner O. et al. (1985). The Nucleoprotein as a Possible Major Factor in Determining Host Specificity of Influenza H3N2 Viruses. Virology 147: 287-294.

Shortridge K.F., Webster R.G., Butterfield W.K. et al. (1977). Persistence of Hong Kong influenza virus variants in pigs. Science 196: 1454-1455.

Scull M.A., Gillim-Ross L., Santos C. (2009). Avian Inlfuenza Virus Glycoproteins Restrict Virus Replication and Spread through Human Airway Epithelium at Temperatures of the Proximal Airways. PLoS Pathogens 5: doi:10.1371/journal.ppat.1000424.

Senne D.A., Panigrahy B., Kawaoka Y. et al. (1996). Survey of the Hemagglutinin (HA) Cleavage Site Sequence of H5 and H7 Avian Influenza Viruses: Amino Acid Sequence at the HA Cleavage Site as a Marker of Pathogenicity Potential. Avian Dis 40: 425-437.

Sha B. and Luo M. (1997). Structure of a bifunctional membrane-RNA binding protein, influenza virus matrix protein M1. Nature Structural Biology 4: 239-244.

Shinya K., Ebina M., Shinya Y. et al. (2006). Influenza virus receptors in the human airway. Nature 440: 435-436.

Shope R. (1931). Swine influenza I. Experimental transmission and pathology. J Exp Med 54: 349-359.

Shortridge K.F., Zhou N.N., Guan Y. et al. (1998). Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 252: 331-342.

Shtyrya Y., Mochalova L., Voznova G. et al. (2009). Adjustment of receptor-binding and neuraminidase substrate specificities in avian-human reassortant influenza viruses. Glycoconj J 26: 99-109.

Shu L.L., Lin Y.P., Wright S.M. et al. (1994). Evidence for Interspecies Transmission and Reassortment of Influenza A Viruses in Pigs in Southern China. Virology 202: 825-833.

Sieczkarski S.B. and Whittaker G.R. (2002). Influenza Virus Can Enter and Infect Cells in the Absence of Clathrin-Mediated Endocytoses. J Virol 76: 10455-10464.

Skehel J. J. and Wiley D. C. (2000). RECEPTOR BINDING AND MEMBRANE FUSION IN VIRUS ENTRY: The Influenza Hemagglutinin. Annual Reviews 69: 531-569.

Smith G.J.D., Vijaykrishna D., Bahl J. et al. (2009a). Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459: 1122-1125.

Smith G.J.D., Bahl J., Vijaykrishna D. et al. (2009b). Dating the emergence of pandemic influenza viruses. PNAS 106: 11709-11712.

Sorrell E.M., Schrauwen E.J.A., Linster M. (2011). Predicting 'airborne' influenza viruses: (trans-) mission impossible? Current Opinion in Virology 1: 635-642.

Spackman E., Gelb J., Preskenis L.A. et al. (2010). The pathogenesis of low pathogenicity H7 avian influenza viruses in chickens, ducks and turkeys. Virol J 7: 331.

Stallknecht D.E. and Shane S.M. (1988). Host range of avian influenza virus in free-living birds. Vet Res Commun 12: 125-141.

Starick E., Lange E., Grund C. et al. (2012). Reassortments of pandemic influenza A virus H1N1/2009 and endemic porcine HxN2 viruses emerge in swine populations in Germany. J Gen Virol 93: 1658-1663.

Steel J., Lowen A.C., Mubareka S. et al. (2009). Transmission of Influenza Virus in a Mammalian Host Is Increased by PB2 Amino Acids 627K or 627E/701N. PLoS Pathogens 5: doi: 10.1371/journal.ppat.1000252.

Steinhauer D.A. (1999). Role of Hemagglutinin Cleavage for the Pathogenicity of Influenza Virus. Virology 258: 1-20

Stevens J., Blixt O , Glaser L. et al. (2006a). Glycan Microarray Analysis of the Hemagglutinins from Modern and Pandemic Influenza Viruses Reveals Different Receptor Specificities. J Mol Biol 355: 1143-1155.

Stevens J., Blixt O., Paulson J.C. et al. (2006b). Glycan microarray technologies: tools to survey host specificity of influenza viruses. Nature Reviews Microbiol 4: 857-864.

Stray S.J., Cummings R.D., Air G.M. (2000). Influenza virus infection of desialylated cells. Glycobiology 10: 649-658.

Suarez D.L. (2000). Evolution of avian influenza viruses. Vet Microbiol 74: 15-27.

Subbarao E.K., London W., Murphy B.R. (1993). A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 67: 1761-1764.

Subbarao K., Murphy B.R., Fauci A.S. (2006). Development of Effective Vaccines against Pandemic Influenza. Immunity 24: 5-9.

Suzuki Y., Nagao Y., Kato H. et al. (1986). Human Influenza A Virus Hemagglutinin Distinguishes Sialyloligosaccharides in Membrane-associated Gangliosides as Its Receptor Which Mediates the Adsorption and Fusion Process of Virus Infection. The J Biol Chem 261: 17057-17061.

Suzuki Y., Ito T., Suzuki T. et al. (2000). Sialic Acid Species as a Determinant of the Host Range of Influenza A viruses. J Virol 74: 11825-11831.

Suzuki Y., Ito T., Suzuki T. et al. (2001). Sialyl sugar chains as receptors and determinants of host range of influenza A viruses. Int Congress Series 1219: 521-255.

Takahashi T., Suzuki Y, Nushinaka D. et al. (2001). Duck and Human Pandemic Influenza A Viruses Retain Sialidase Activity under Low pH Conditions. J Biochem 130: 279-283.

Tamura S. and Kurata T. (2004). Defense Mechanisms against Influenza Virus Infection in the Respiratory Tract Mucosa. Jpn J Infect Dis 57: 236-247.

Tamura S. and Kurata T. (2005). Mechanisms of Broad Cross-Protection Provided by Influenza Virus Infection and Their Application to Vaccines. Jpn J Infect Dis: 58 195-207.

Taubenberger J.K., Reid A.H., Lourens R.M. et al. (2005). Characterization of the 1918 influenza virus polymerase genes. Nature 437: 889-893.

Taubenberger J.K. and Morens D.M. (2006). 1918 influenza: the mother of all pandemics. Emerg Infect Dis 12: 15-22.

Thompson C.I., Barclay W.S., Zambon M.C. et al. (2006). Infection of Human Airway Epithelium by Human and Avian Strains of Influenza A Virus. J Virol 80: 8060-8068.

Toffan A., Beato M.S., De Nardi R. et al. (2008). Conventional inactivated bivalnet H5/H7 vaccine prevents viral localization in muscles of turkeys infected experimentally with low pathogenic avian influenza and highly pathogenic avian influenza isolates. Avian Pathol 37: 407-412.

Tong S., Li Y., Rivailler P. et al. (2012). A distinct lineage of influenza A virus from bats. PNAS: doi:10.1073/pnas.1116200109.

Trebbien R., Larsen L.E., Viuff B.M. (2011). Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virol J: doi:10.1186/1743-422X-8-434.

Trifonov V., Khiabanian H., Rabadan R. (2009). Geographic Dependence, Surveillance, and Origins of the 2009 Influenza A (H1N1). Virus New England Journal of Medicine: doi: 10.1056/NEJMp0904572.

Tumpey T.M., Kapczynski D.R., Swayne D.E. (2004). Comparative Susceptibility of Chickens and Turkeys to Avian Influenza A H7N2 Virus Infection and Protective Efficacy of a Commercial Avian Influenza H7N2 Virus Vaccine. Avian Dis 48: 167-176.

Tumpey T.M., Maines T.R., Van Hoeven N. et al. (2007). A Two-Amino Acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science 315: 655-659.

van Eijk M., White M.R., Crouch E.C. et al. (2003). Porcine Pulmonary Collectins Show Distinct Interactions with Influenza A Viruses; Role of the N-linked Oligosaccharides in the Carbohydrate Recognition Domain. J Immunol 171: 1431-1440.

van Eijk M., White M.R., Batenburg J.J. et al. (2004). Interactions of Influenza A Virus with Sialic Acids Present on Porcine Surfactant Protein D. Am J Respir Cell Mol Biol 30: 871-879.

Van Hoeven N., Pappas C., Belser J.A. et al. (2009). Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air. PNAS 106: 3366-3371.

Van Kerkhove M.D., Mumford E., Mounts A.W. et al. (2011). Highly pathogenic avian influenza (H5N1): pathways of exposure at the animal-human interface, a systemic review. PLoS ONE 6: doi:10.1371/journal.pone.0014582

Van Poucke S.G.M., Nicholls J.M., Nauwynck H.J. et al. (2010). Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. Virol J 7: doi:10.1186/1743-422X-7-38.

Van Reeth K., Van Gucht S., Pensaert M. (2002). Correlations between lung proinflammatory cytokine levels, virus replication, and disease after swine influenza virus challenge of vaccination-immune pigs. Vir Immun 15: 583-594.

Van Reeth K., Brown I.H., Dürrwald I. et al. (2008). Seroprevalence of H1N1, H3N2 and H1N2 viruses in pigs in seven European countries in 2002-2003. Influenza Other Resp Viruses 2: 99-105.

Van Reeth K., Braeckmans D., Cox E. et al. (2009). Prior infection with an H1N1 swine influenza virus partially protects pigs against low pathogenic H5N1 avian influenza virus. Vaccine 27: 6330-6339.

Van Reeth K., Brown I.H., Olsen C.W. (2012). Swine Influenza. In: Zimmerman J.J., Kamiker L.A., Ramirez A. et al. (Eds) Diseases of Swine, John Wiley & Sons Inc., Chichester, West Sussex, UK, 557-571.

Van Riel D., Munster V.J., de Wit E. et al. (2007). Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am J Pathol 171: 1215-1223.

Van Riel D., den Bakker M.A., Leijten L.M. et al. (2010). Seasonal and pandemic human influenza viruses attach better to human upper respiratory tract than avian influenza viruses. Am J Pathol 176: 1614-1618.

Varki A. (1997). Sialic acids as ligands in recognition phenomena. FASEB J 11: 248-255.

Varki A. (2001). Loss of N-Glycolylneuraminic Acid in Humans: Mechanisms, Consequences, and Implications for Hominid Evolution. Yearbook of Physical Anthropology 44: 54-69.

Vigerust D.J., Ulett K.B., Boyd K.L. et al. (2007). N-Linked Glycosylation Atttenuates H3N2 Influenza Viruses. J Virol 81: 8593-8600.

Vines A., Wells K., Matrosovich M. et al. (1998). The role of influenza A virus Hemagglutinin residues 226 and 228 in Receptor Specificity and host range restriction. J Virol 72: 7626-7631.

von Itzstein M. (2007). The war against influenza: discovery and development of sialidase inhibitors. Nature Reviews Drug Discovery 6: 967-974.

Wang H., Feng Z., Shy Y. et al. (2008). Probable limited person-to-person transmission of highly pathogenic avian influenza A (H5N1) virus in China. The Lancet 371: 1427-1434.

Wan H. and Perez D.R. (2006). Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. Virology 346: 278-286.

Wan H. and Perez D.R. (2007). Amino Acid 226 in the Hemagglutinin of H9N2 Influenza Viruses Determines Cell Tropism and Replication in Human Airway Epithelial Cells. J Virol 81: 5181-5191.

Wan H., Sorell E.M., Song H. et al. (2008). Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS ONE 13: doi:10.1371/journal.pone.0002923.

Watanabe T., Watanabe S., Kawaoka Y. (2010). Cellular Networks Involved in the Influenza Virus Life Cycle. Cell Host & Microbe 7: 427-439.

Webby R.J., Swenson S.L., Krauss S.L. et al. (2000). Evolution of Swine H3N2 Influenza Viruses in the United States. J Virol 74: 8243-8251.

Webster R.G., Yakhno M., Hinshaw V.S. et al. (1978). Intestinal Influenza: Replication and Characterisation of Influenza Viruses in Ducks. Virology 84: 268-278.

Webster R.G., Bean W.J., Gorman O.T. et al. (1992). Evolution and Ecology of Influenza A Viruses. Microbiol Rev 56: 152-179.

Webster R.G. and Bean J.W.J. (1998). Evolution and Ecology of Influenza Viruses: Interspecies Transmission. In: Nicholson K.G., Webster R.G., Hay A.J. (Eds) Textbook of Influenza. Blackwell Science Ltd., Malden, MA, USA, p. 109-119.

Wertheim J.O. (2010). The Re-Emergence of H1N1 Influenza Virus in 1977: A Cautionary Tale for Estimating Divergence Times Using Biologically Unrealistic Sampling Dates. PLoS ONE 5: doi:10.1371/journal.pone.0011184.

Wiley D.C., Wilson I.A., Skehel J.J. (1981). Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their infvolvement in antigenic variation. Nature 289: 373-378.

Wise H.M., Foeglein A., Sun J. et al. (2009). A Complicated Message: Identification of a Novel PB1-Related Protein Translated from Influenza A Virus Segment 2 mRNA. J Virol 83: 8021-8031.

Whittaker R.G. and Digard P. (2006). Entry and intracellullar transport of influenza virus. In: Kawaoka Y. (Ed) Influenza virology: current topics. Caister Academic Press, Wymondham, Norfolk, England, p. 37-64.

Whittle J.R.R., Zhang R., Khurana S. et al. (2011). Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. PNAS 108: 14216-14221.

Wood G.W., Parsons G., Alexander D.J. (1995). Replication of influenza A viruses of high and low pathogenecity for chickens at different sites in chickens and ducks following intranasal inoculation. Avian Pathol 24: 545-551.

Woo G.H., Kim H.J., Bae Y.C. et al. (2011). Comparative histopathological characteristics of highly pathogenic avian influenza (HPAI) in chickens and domestic ducks in 2008 Korea. Histol Histopathol 23: 167-175.

Wright P.F. and Webster R.G. (2001). Orthomyxoviruses. In: Knipe D.M and Howley P.M. (Eds) Fields Virology. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, USA, p. 1533-1579.

Yamada S., Hatta M., Staker B.L. et al. (2010). Biological and Structural Characterization of a Host-Adapting Amino Acid in Influenza Virus. PLoS Pathogens 6: doi:10.1371/journal.ppat.1001034.

Yamnikova S.S., Gambaryan A.S., Tuzikov A.B. et al. (2003). Differences Between HA Receptor-Binding Sies of Avian Influenza Viruses Isolated from Laridae and Anatidae. Avian Dis 47: 1164-1168.

Yao L., Korteweg C., Hsueh W. et al. (2008). Avian influenza receptor expression in H5N1-infected and noninfected human tissues. FASEB J 22: 734-740.

Yen H., Liang C., Wu C. et al. (2011). Hermagglutinin-neuraminidase balance confers respiratory-droplet transmissibility of the pandemic H1N1 influenza virus in ferrets. PNAS 108: 14264-14269.

Yu H., Zhou Y., Li G. et al. (2011). Genetic diversity of H9N2 influenza viruses from pigs in China: A potential threat to human health? Vet Microbiol 149: 254-261.

Zell R., Motzke S., Krumbholz A. et al. (2008). Novel reassortant of swine influenza H1N2 virus in Germany. J Gen Virol 89: 271-276.

Zeng H., Goldsmith C.S., Maines T.R. et al. (2013). Tropism and Infectivity of Influenza Virus, Including Highly Pathogenic Avain H5N1 Virus, in Ferret Tracheal Differentiated Primary Epithelial Cell Cultures. J Virol 87: doi: 10.1128/JVI.02885-12.

Zhang J., Pekosz A., Lamb R.A. (2000). Influenza virus assambly and lipid raft microdomains: a role for the cytoplasmatic tails of the spike glycoproteins. J Virol 74: 4634-4644.

Zhang P., Tang Y., Liu X. et al. (2009). A Novel Genotype H9N2 Influenza Virus Possessing Human H5N1 Internal Genomes Has Been Circulating in Poultry in Eastern China since 1998. J Virol 83: 8428-8438.

Zhou J., Law H.K.W., Yan Cheung C. et al. (2006). Differential Expression of Chemokines and Their Receptors in Adult and Neonatal Macrophages Infected with Human or Avian Influena Viruses. J Inf Dis 194: 61-70.

Zhou N.N, Senne D.A., Landgraf J.S. et al. (1999). Genetic Reassortment of Avian, Swine, and Human Influenza A Viruses in American Pigs. J Virol 73: 8851-8856.

CHAPTER 2.

AIMS OF THE THESIS

Since the 1980s it has been proposed that pigs play an important role in human influenza ecology, functioning either as an intermediate host for the adaptation of avian influenza viruses to mammals or as a mixing vessel for the generation of reassortant viruses. Earlier results obtained in our laboratory as well as by other research groups, revealed that the molecular determinants that control the host range restriction of influenza viruses in mammals and birds are not well identified. In contrast with earlier findings, domestic pigs did not seem to be highly susceptible to infection with AIVs nor did pig-to-pig transmission of both low- and highly-pathogenic AIVs occur (efficiently) under experimental conditions. Unraveling the viral factors that determine the host range is of the uttermost importance for the early detection of potential pandemic viruses during surveillance programs. Out of thousands of influenza virus isolates collected each year in animal surveillance studies, how do we identify the dangerous ones? To this date, as illustrated by the 2009 outbreak of pandemic H1N1, the scientific community lacks the capacity to predict/identify potential emerging influenza viruses. The expansion in surveillance efforts in pigs, in response to the latest pandemic, will most probably identify many more reassortant viruses in this host, but will it be possible to select the ones we should prepare for as pandemic threats?

The specific aims of this thesis were:

- ✓ To study influenza virus-host interactions in more detail, under well controlled conditions and with a reduction of experimental animals, by the development of porcine explants systems. The *ex vivo* model had to cover both the porcine upper and lower respiratory tract, showing maximal similarities with the *in vivo* situation and being susceptible to infection with influenza A viruses. Simultaneously, a comparative and detailed study of the expression of receptor variants in the porcine respiratory tract *in vivo* and *ex vivo* was conducted. The former was necessary to assure that receptor expression was not changed due to cultivation *in vitro* (chapter 3).

- ✓ To compare replication, tissue tropism and transmission in pigs between two influenza viruses with a distinct receptor binding properties. We used reverse genetics-generated viruses differing solely by two amino acids in the receptor binding site of their hemagglutinin. One virus was the wild-type A/Hong Kong/1/68 H3N2 (R1-HK), the other virus (R2-HK) contained L226Q and S228G substitutions in its HA. The latter mutations are known to result in an avian-like receptor binding preference. First, we studied the replication potential of

these viruses in the explants systems, challenging the system for its sensitivity. Next, the transmissibility and organ tropism of these viruses were studied *in vivo* (Chapter 4.1.).

- ✓ To find an explanation for the lack of pig-to-pig transmission of the virus with the avian-like receptor specificity (R2-HK) observed in chapter 4.1. We examined the quantity of HA expressed on the virions and the release of the viruses from MDCK-cells. Furthermore, we studied the cell tropism of R1-HK and R2-HK *in vivo* and *ex vivo* as well as their interactions with mucus and surfactant proteins-D and -A (chapter 4.2.).

- ✓ Finally, to study if any of the 5 additional mutations in the hemagglutinin of A/Hong Kong/1/68 (H3N2), observed at the moment of introduction in the human population, were contributing to the adaptation to a mammalian host. Therefore R5-HK, containing I62R, N81D, K92N, G144A and S193N mutations, was assessed for its replication capacity *ex vivo* and its transmissibility between pigs. A mutant virus with an additional D60G mutation in the HA1, which arose during the transmission experiment, was phenotypically characterized (chapter 5).

CHAPTER 3.

REPLICATION OF AVIAN, HUMAN AND SWINE INFLUENZA VIRUSES IN PORCINE RESPIRATORY EXPLANTS AND ASSOCIATION WITH SIALIC ACID DISTRIBUTION

Van Poucke S.G., Nicholls J.M., Nauwynck H.J., Van Reeth K.

Virology Journal (2010), 7:38

3.1. Abstract

Throughout the history of human influenza pandemics, pigs have been considered the most likely "mixing vessel" for reassortment between human and avian influenza viruses (AIVs). However, the replication efficiencies of influenza viruses from various hosts, as well as the expression of sialic acid (Sia) receptor variants in the entire porcine respiratory tract have never been studied in detail. Therefore, we established porcine nasal, tracheal, bronchial and lung explants, which cover the entire porcine respiratory tract with maximal similarity to the in vivo situation. Subsequently, we assessed virus yields of three porcine, two human and six AIVs in these explants. Since our results on virus replication were in disagreement with the previously reported presence of putative avian virus receptors in the trachea, we additionally studied the distribution of sialic acid receptors by means of lectin histochemistry. Human (Siaα2-6Gal) and avian virus receptors (Siaα2-3Gal) were identified with Sambucus nigra and Maackia amurensis lectins respectively.

Compared to swine and human influenza viruses, replication of the AIVs was limited in all cultures but most strikingly in nasal and tracheal explants. Results of virus titrations were confirmed by semi-quantification of infected cells using immunohistochemistry. By lectin histochemistry we found moderate to abundant expression of the human-like virus receptors in all explant systems but minimal binding of the lectins that identify avian-like receptors, especially in the nasal, tracheal and bronchial epithelium.

The species barrier that restricts the transmission of influenza viruses from one host to another remains preserved in our porcine respiratory explants. Therefore this system offers a valuable alternative to study virus and/or host properties required for adaptation or reassortment of influenza viruses. Our results indicate that, based on the expression of Sia receptors alone, the pig is unlikely to be a more appropriate mixing vessel for influenza viruses than humans. We conclude that too little is known on the exact mechanism and on predisposing factors for reassortment to assess the true role of the pig in the emergence of novel influenza viruses.

3.2. Introduction

Pigs are important natural hosts for influenza A viruses, which are a major cause of acute respiratory disease. Influenza viruses of H1N1, H3N2 and H1N2 subtypes are enzootic in swine populations worldwide. Most of these swine influenza viruses are the product of genetic reassortment between viruses of human and/or avian and/or swine origin and their phylogeny and evolution are complex (Castrucci et al. 1993, Brown et al. 1998, Zhou et al. 1999). The swine influenza viruses circulating in Europe have a different origin and antigenic constellation than their counterparts in North America or Asia and within one region multiple lineages of a given subtype can be present (Brown 2000, Olsen et al. 2006). Although natural infections of pigs with avian (Guan et al. 1996, Karasin et al. 2000, Peiris et al. 2001, Ninomiya et al. 2002, Karasin et al. 2004) or human influenza viruses (Karasin et al. 2000b, Subbarao et al. 2006) also occur, these viruses were rarely capable of establishing themselves as a stable lineage in pigs without undergoing genetic adaptation (Klenk et al. 2008).

Based on such epidemiological observations and on the close interactions that take place between pigs and birds in Asia, Scholtissek was the first to propose pigs as intermediate hosts or as mixing vessels for reassortment (Scholtissek 2009, Ma et al. 2009). Because sialic acids (Sia) bound to galactose with α2-6 and α2-3 linkages (receptors preferred by human and avian influenza viruses respectively) were identified in the porcine trachea (Ito et al. 1998, Suzuki et al. 2000, Wan and Perez 2006) this was considered the molecular proof of the mixing vessel hypothesis . As such, co-infection with human and AIVs or with human, swine and AIVs could lead to the emergence of new influenza viruses with a pandemic potential. On the other hand, the generation of pandemic influenza viruses in pigs appears to be a rare and complex process, and the 2009 H1N1 influenza virus is the first pandemic virus that is almost certainly of swine origin.

Though experimental *in vivo* studies (Kida et al. 1994, Hinshaw et al. 1998, Shortridge et al. 1998, Landolt et al. 2003, Choi et al. 2005) have confirmed the susceptibility of pigs to both avian and human influenza viruses, they also point towards a strong species barrier as virus titers obtained from the respiratory tract and from nasal swabs were invariably lower for the heterologous viruses than for typical swine influenza viruses. In addition, all AIVs examined failed to transmit between pigs (Loeffen et al. 2004, De Vleeschauwer et al. 2009). Limited *ex vivo* or *in vitro* studies, using either porcine tracheal organ cultures (Schmidt et al. 1974) or primary swine respiratory epithelial cell cultures (SRECs) (Busch et al. 2008) confirmed the lower susceptibility of the pig tissues to most heterologous viruses. In the SRECs, Busch and co-workers identified molecular differences in the HA gene which correlated with the divergence in infectivity.

However, the replication efficiencies of influenza viruses from various hosts as well as the expression of Sia receptor variants have never been compared at all levels of the porcine respiratory tract. For

this purpose, we (1) established porcine nasal, tracheal, bronchial and lung explants covering the entire porcine respiratory tract with maximal similarity to the *in vivo* situation, (2) investigated the replication ability of avian, human and swine influenza viruses in all relevant parts of the respiratory tract and (3) analyzed the receptor distribution by means of lectin histochemistry.

3.3. Methods

3.3.1. Animals

Five 6-week-old pigs from a high health status farm that was negative for influenza A viruses were used. The animals were housed together in a HEPA-filtered experimental unit with ad libitum access to water and food. At arrival they were treated intramuscularly with ceftiofur (Naxcel®, Pfizer-1 ml/20 kg body weight) to clear the respiratory tract from possible infections with *Actinobacillus pleuropneumoniae, Pasteurella multocida, Haemophilus parasuis* and *Streptococcus suis*. Two days later they were euthanized by intravenous administration of thiopental (Penthotal®, Kela-12.5 mg/kg body weight) and exsanguinated.

3.3.2. Isolation and culture of respiratory explants

To cover both the upper and lower respiratory tract, four different systems were used: nasal (NE), tracheal (TE), bronchial (BE) and lung explants (LE).

Nasal explants

The NE were cultivated according to the air-liquid interface principle. NE were prepared as described by Glorieux et al. (2007). In short, the respiratory mucosa was carefully stripped from the medial side of the ventral turbinates and from the nasal septum. This tissue was cut in squares of 25 mm^2 each, which were transferred to fine meshed gauzes in 6-well plates with the epithelium facing up. Each well contained two ml of medium ((50% DMEM (Gibco)/50% RPMI (Gibco), penicillin 100U/ml (Gibco), streptomycin 100µg/ml (Gibco), gentamycin 0.1 mg/ml (Gibco), glutamine 0.3 mg/ml (BDH Biochemical)) so the epithelium was slightly immersed in fluid. Explants were cultured in an incubator at 37 °C and 5% CO_2.

Tracheal explants

The trachea was excised distal from the larynx and proximal to the bifurcation. This part was divided in two by a sagittal incision and both halves were pinned onto a sterile board so the adventitia and cartilage could be removed. The remaining tissue (mucosa with some submucosa) was then cut in

pieces of 25 mm^2 and processed similar to the nasal mucosa. Cultivation also took place following the air-liquid interface principle.

Bronchial explants

The left lung was removed from the thorax and placed into transport medium (phosphate buffered saline (PBS), penicillin 1000U/ml (Gibco), streptomycin 1 mg/ml (Gibco), gentamycin 0.5 mg/ml (Gibco), amphotericin B 5 mg/ml (fungizone®, Bristol-Myers)). Next the surrounding lung tissue was manually dissected out until only the bronchial tree remained. Bronchial rings of approximately two mm in diameter and three mm long were cut. These rings were transferred to 16 ml capped culture tubes containing one ml of medium (MEM (Gibco), penicillin 100 U/ml (Gibco), streptomycin 100 µg/ml (Gibco), kanamycin 1 µg/ml (Gibco), glutamine 0.3 mg/ml (BDH Biochemical), HEPES 0,02 M/ 100 ml (Gibco)). To imitate the in vivo situation, explants were alternately exposed to air and medium by putting them at 37 °C in a slowly turning device (0.5 turn/minute) for rotating culture tubes.

Lung explants

Thin LE were obtained following a technique described earlier for rat lung explants (Porro et al. 2001), with slight modifications. We opened the thorax in the ventral midline and placed a ligature on the left trachea bifurcation. Two canules, each connected to a 20 ml syringe, were advanced down the right trachea bifurcation. One syringe contained cold PBS, the other air. As such the right lung was simultaneously perfused and aerated in situ. In the laminar flow the right apical lobe was fully expanded by inflating a 1% agarose solution. The agarose (type VII-A low gelling temperature, Sigma) had been dissolved in white PBS, autoclaved, microwave heated and cooled down to 37 °C. The expanded lung was placed at 4 °C for 10 minutes in a sterile container until the agarose solidified. This tissue was then cut up in cubes with a cross section of one cm^2, which were transferred to a 20 ml syringe with seven ml of 4% agarose. After replenishing the syringe with more agarose it was placed at 4 °C for 15 minutes. The embedded lung tissue was cut in slices of one mm thick, using a cryotome blade. These slices were trimmed until they had a surface of 25 mm^2 and incubated overnight in 24-well plates with one ml of medium (DMEM (Gibco), bovine insulin 2.5 µg/ml (Sigma), hydrocortisone 0.5 µg/ml (Sigma), vitamin A 0.5 µg/ml (Sigma), gentamycin 0.1 mg/ml (Gibco)) at 37 °C and 5% CO_2. After 24 hours the explants were thoroughly washed with warm PBS to remove the remaining agarose. Finally they were transferred to 6-well plates with two ml of medium and cultured at 37 °C and 5% CO2.

3.3.3. Analysis of viability

To evaluate virus yields over time we had to confirm that fluctuations in virus titers did not result from a decreased explant viability. Nasal and tracheal explants were checked daily for ciliary beating by light microscopy.

At 0, 24, 48, 72 and 96 hours post culture two explants of each system were collected. One was used to determine the percentage of necrotic cells by an EMA staining (Invitrogen), the other to determine the percentage of apoptotic epithelial cells using an "In Situ Cell Death Detection Kit" (Roche) (Darzynkiewicz et al. 1997). The latter is based on Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling (TUNEL) to detect DNA strand breaks. The EMA staining was performed on non fixed tissues, the TUNEL staining on cryostat sections. From each explant 12 cryosections, dispersed over the entire sample, were cut. For each section the positive cells within one ad random selected microscopic field (magnification 1000x) were counted. The number of epithelial cells in the NE, TE and BE as well as the total number of cells in the LE were determined by staining the nuclei with Hoechst (1:100 diluted in ultra pure water (U.P.)) (Invitrogen).

EMA

The explants were transferred to a 24-well plate and washed once with medium. They were incubated in the dark with 300 µl ethidium monoazide bromide (EMA) (1:20 diluted in medium) for one hour at 4 °C. Next they were exposed to a bright light source for 10 minutes. After three more washes with medium, the explants were embedded in methylcellulose (Methocel®, Fluka) and preserved at -70 °C. Afterwards six µm thick sections were methanol fixed during 20 minutes at -20 °C and counterstained with Hoechst (1:100 in U.P.) (Invitrogen).

TUNEL

The TUNEL reaction was performed according to the manufacturer's instructions. The explants were embedded in methylcellulose, cut into 12 slices of six µm thick, methanol fixed and counterstained with Hoechst.

3.3.4. Viruses, inoculation and evaluation of virus replication

Three porcine, two human and six AIVs were used (overview Table 1). The human and porcine influenza strains were representatives of viruses that are currently widespread in the human or swine population. Despite numerous passages of the pig viruses in eggs, no mutations were present in their receptor binding site. For all human and porcine strains, the virus titers of the stocks were determined both on eggs and MDCK cells, revealing no big differences between the two methods. In

general titers obtained on MDCK cells were slightly higher than those on eggs, indicating that none of these viruses were strongly egg adapted. The avian viruses were low pathogenic isolates from both *Galliformes* and *Anseriformes* and belonged to different HA subtypes.

At least three repeats were conducted for each explant system and each virus subtype. After 24 hours of culture and one washing step with PBS, the explants were inoculated with 10^6 EID_{50} virus in a volume of 600 µl. For this purpose, NE, TE and LE were transferred to 24-well plates while the BE remained in the cell culture tubes. One hour of incubation with the inoculum at 37 °C was followed by three subsequent washing steps with 0.5 ml warm PBS so non-attached viruses were removed. Next the explants were placed back in the original 6-well plates or culture tubes with 2.3 ml (NE, TE and LE) or 1.3 ml (BE) of new medium.

To assess virus yields, 300 µl of supernatant was collected at 1, 24 and 48 hours post inoculation. Ten-fold serial dilutions of the supernatant were inoculated onto MDCK cells grown in 96-well plates. These plates were incubated for seven days at 37 °C with 5% CO_2 and checked for cytopathogenic effect. The presence of virus replication in each well was confirmed by an immunocytochemical staining, which was analyzed by light microscopy. The cells, fixed with paraformaldehyde 4% for 10 minutes at room temperature, were incubated with mouse anti-NP monoclonal HB-65 antibody (1:50, ATCC) for two hours. Subsequently, incubation with horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody (1:200, Dako) for one hour was followed by a development step with H_20_2 as substrate and 3-amino-9-ethyl-carbazole (AEC) as precipitating agent. Virus titers were calculated by the method of Reed and Münch (1938) and expressed as $TCID_{50}$/ml. Statistical analysis to compare the titers of the avian, the swine and human viruses in Figure 1 was carried out using the Kruskal-Wallis test with a 95 % confidence interval ($p<0.05$). The avian and swine viruses were compared as groups at 24 and 48 hpi, the human viruses were compared separately because of the consistent differences in virus yield between the 2 subtypes.

Because the virus titers obtained with AIVs were frequently low, additional controls were performed to confirm that these titers resulted from productive virus infection rather than from the release of input virus immediately after the attachment step. To this purpose a parallel experiment was performed with explants that only allow attachment of the virus and no virus entry or subsequent steps (Ohuchi et al. 1997). One NE, TE, BE and LE were prepared as described above. After 24 hours they were fixed in one ml of 1% paraformaldehyde at 4 °C for one hour. Next they were extensively washed with PBS and inoculated with 600 µl 10^6 EID_{50} Chicken/Belgium/150/99 (H5N2). After one hour of incubation at 37 °C the inoculum was removed, the explants were washed three times with PBS and the medium was renewed. Supernatant was again collected at 1, 24 and 48 hpi.

Table 1. Summary of the influenza viruses used for inoculation of the explants. These viruses were kindly provided to us by:* Alan Hay, National Institute for Medical Research, London, U.K./ † Robert Webster, St. Jude Children's Research Hospital, Memphis, Tennessee, U.S.A./ ‡ Thierry van den Berg, Veterinary and Agrochemical Research Centre, Brussels, Belgium/ § Ilaria Capua, Instituto Zooprofillatico delle Venezie, Legnaro, Padova, Italy

Influenza virus	Number of passages in embryonated eggs
Swine influenza viruses:	
Sw/Belgium/1/98 (H1N1)	3
Sw/Flanders/1/98(H3N2)	3
Sw/Gent/7625/99 (H1N2)	3
Human influenza viruses:	
A/New Caledonia/20/99 (H1N1) *	4
A/Panama/2007/99 (H3N2) *	7
Low pathogenic AIVs:	
Duck/Italy/1447/05 (H1N1) §	4
Mallard/Alberta/279/98 (H3N8) †	3
Duck/Belgium/06936/05 (H4N6) ‡	3
Chicken/Belgium/150/99 (H5N2) ‡	3
Chicken/Italy/1067/V99 (H7N1) §	4
Mallard/Italy/3401/2005 (H5N1) §	3

3.3.5. Evaluation of the dose response

Additionally we wished to examine the effect of the inoculation dose on the yield of swine and AIVs at different levels of the respiratory tract. Therefore NE, TE, BE and LE were inoculated with Swine/Gent/7625/99 (H1N2), Duck/Belgium/06936/05 (H4N6) and Chicken/Belgium/150/99 (H5N2) at three different doses: 10^6, 10^5 and 10^4 EID_{50} virus in a volume of 600 µl. Collection and titration of the supernatant was performed as described above. Each condition was repeated twice.

3.3.6. Influenza A nucleoprotein detection

Since virus titrations of the supernatant do not provide information on the number or type of infected cells, we fixed the explants inoculated with Swine/Gent/7625/99 (H1N2) and Duck/Belgium/06936/05 (H4N6) at 48 hpi to perform immunohistochemistry (IHC) and immunofluorescense (IF).

IHC was carried out on formalin fixed (for 24 hours) and paraffin embedded explants. Ten consecutive sections of 4 µm thick were cut in six different areas of each explant. Because formalin-fixation can cause protein cross-linking, antigen retrieval (AR) was applied to stain the viral

nucleoprotein. Enzyme-induced AR was accomplished by incubating the deparaffinized and rehydrated sections with 0.1% pronase (Roche) at 37 °C for four minutes. Endogenous peroxidase and biotin activity were blocked by incubation with 3% H_2O_2 and the biotin/avidin blocking kit (Vector) at room temperature respectively. Incubation with the anti-influenza A nucleoprotein monoclonal antibody (HB65, ATT) at a 1:400 dilution was performed at room temperature for 90 minutes. Slides were rinsed three times in 0.05 mol/L Tris-buffer and incubated with biotin-conjugated rabbit anti-mouse immunoglobulin at a 1:100 dilution (Dako) for 30 minutes at room temperature. After another wash they were incubated with the ABC-complex for 30 minutes, developed with AEC (Vector developing kit) and counterstained with hematoxylin.

To exclude that AR gives rise to false positive results, we compared the localization of virus positive cells after IHC with those after IF. Therefore similar explants were embedded in Methocel® (Sigma), frozen at -70 °C and entirely cut in sections of 8 µm thick. These sections were fixed in methanol for 10 minutes at -20 °C. Since this fixation method only causes precipitation of proteins, AR was not required. The anti-influenza A nucleoprotein monoclonal antibody was incubated at a 1:50 dilution followed by FITC labeled goat-anti mouse IgG (Molecular Probes) at a 1:200 dilution, both for one hour at 37 °C.

Semi quantitative information on the infectivity was obtained by IF on cryosections, where the following scoring system was applied: -: no virus positive epithelial cells, +/-: single positive cells covering <10% of the epithelium, +: between 11 and 40% of the epithelium is positive, ++: between 41 and 70% of the epithelium is positive, +++: between 71 and 100% of the epithelium is positive.

3.3.7. Lectin histochemistry

The distribution of α2-3 and α2-6 linked Sia receptors in explants, 24 hours post culture, was detected by lectin histochemistry. The tissues had been fixed in 4% buffered formalin during 24 hours and were paraffin embedded. In an identical manner fresh nasal, tracheal, bronchial and lung tissues were analyzed to check for the effects of cultivation on receptor expression. Because formalin fixation can cause protein cross-linking, thereby hiding antigenic sites, antigen retrieval (AR) was applied. Nicholls et al. (2007) have previously shown that heat induced AR by microwaving the samples in 10 mM citrate buffer pH 6.0 at 95 °C for 15 minutes is the optimal method for the retrieval of Sia receptors. To exclude false positive results by AR, some control sections were pretreated with neuraminidase (DAS 181) removing the Sia. In these stainings we obtained negative results, showing that the AR unmasked only the epitope of interest.

Duck intestines, which only contain Siaα2-3Gal linkages, were used as a control for the specificity of the MAA and SNA lectins.

Expression of α2-6 linked Sia

The α2-6 distribution was examined using a digoxigenin labelled Sambucus nigra agglutinin (SNA) used at a 1:200 dilution (Roche). Sections were incubated for one hour at room temperature. Subsequently, the slides were incubated with 1:200 sheep alkaline phosphatase conjugated anti-digoxigenin Fab fragments (Roche) and developed with New Fuchsin (Dako).

Expression of α2-3 linked Sia

Two different isoforms of the Maackia amurensis agglutinin (MAA), MAA-I and MAA-II that both bind to the α2-3-linked Sia moiety of the receptor, were used. They do differentiate though at the next glycoside level between galactose β1-4 or β1-3 linkages respectively (Brinkman-Van der Linden et al. 2002). The biotinylated MAA-I and -II (Vector Laboratories), both 1:200 diluted, were incubated overnight at 4 °C. Staining was developed with a strep-ABC complex 1:100 (DakoCytomation) and an AEC-substrate kit (Vector Laboratories). Subsequent sections were also stained with digoxigenin conjugated MAA from Roche, as used by Ito and colleagues (1998), together with digoxigenin conjugated MAA-I and MAA-II. The latter were obtained using the Dig Conjugation Kit (Roche) and aimed to examine if the conjugation method can influence the lectin binding.

3.4. Results

3.4.1. Viability

The cilia on the epithelial cells of the nasal explants (NE) and tracheal explants (TE) continued beating for at least 72 h after sampling.

The percentages of ethidium monoazide bromide (EMA) and Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling (TUNEL) positive cells in the four explant systems between 0 and 96 hours post culture (hpc) are shown in Table 2. Every result was the mean of 12 counts. The percentage of necrotic and apoptotic cells generally remained below 5% for NE and TE and below 10% for bronchial explants (BE) and lung explants (LE) during the entire period. There were only two exceptions: the TE at 24 hpc and the LE at 96 hpc.

Overall, these results indicated that the fluctuations of virus yields over time were a true reflection of virus replication and not the consequence of a reduced explant viability (since the proportion of dead cells in the explants showed little variation until at least 72 hpc).

Table 2. Viability of explant systems Mean percentages of apoptotic (TUNEL stained) and necrotic (EMA stained) cells in the four explant systems until 96 hours post cultivation.

	% EMA-positive cells at…..h of cultivation (sd)				
	0	24	48	72	96
NE	0.3 ± 0.6	0.9 ± 1.4	0.2 ± 0.6	0.7 ± 0.6	0.5 ± 0.9
TE	1.9 ± 1.4	0.6 ± 1.0	0.8 ± 1.5	0.8 ± 0.4	0.6 ± 1.1
BE	1.7 ± 1.3	5.2 ± 1.8	1.6 ± 1.6	5.0 ± 2.1	3.0 ± 1.9
LE	5.1 ± 2.8	4.4 ± 1.3	5.1 ± 2.4	5.1 ± 2.5	7.7 ± 1.4
	% TUNEL-positive cells at…..h of cultivation (sd)				
	0	24	48	72	96
NE	0.8 ± 1.0	0.5 ± 0.7	0.5 ± 0.7	0.8 ± 0.8	0.8 ± 1.0
TE	1.1 ± 1.3	5.0 ± 2.1	0.9 ± 1.2	1.0 ± 1.4	1.1 ± 1.1
BE	5.3 ± 1.6	5.0 ± 1.0	5.0 ± 2.1	3.0 ± 1.7	5.1 ± 1.1
LE	3.7 ± 1.4	5.1 ± 1.2	5.0 ± 0.7	5.3 ± 1.6	10.0 ± 1.4

3.4.2. Virus yield

All swine, human and avian isolates yielded infectious virus in the four explant systems. As shown in Figure 1, virus titers in the supernatant were significantly higher at 24 than at 1 hpi. The virus titers of Chicken/Belgium/150/99 in the supernatant of fixed explants, non permissive to infection, were at or below the detection limit by 48 hpi. This indicates that the titers of the AIVs by 48 hpi, although low in NE and TE, most likely are the result of a limited replication.

Swine influenza viruses

The three porcine influenza subtypes replicated most efficiently in the NE, TE and BE with still increasing virus yields between 24 and 48 hpi. At 48 hpi there were minimal differences in virus titers between the various subtypes. In these explants, the swine influenza viruses reached higher virus titers than any of the heterologous viruses, except for A/Panama/2007/99 (H3N2). In the LE, the replication capacity of the swine influenza viruses was more similar to that of the human and avian influenza viruses and somewhat lower than in the other explants.

Human influenza viruses

The two human isolates showed a clear distinction in their replication efficiency. In the NE, TE and BE A/Panama/2007/99 (H3N2) behaved similar to the swine influenza viruses, while the virus titers of A/New Caledonia/20/99 (H1N1) were in between those of the swine and avian strains. The virus titers of both subtypes were highest in the BE and, as for the swine influenza viruses, lower in the LE.

Avian influenza viruses

Of the heterologous viruses, the group of AIVs was least successful in replication and the only one with lower virus titers at 48 hpi than at 24 hpi in some cases. The differences in titers between the avian and swine influenza viruses were most pronounced in the NE and TE. While at 48 hpi, the maximum AIV titer reached 3.1 log $TCID_{50}$/ml in the NE, the minimum titer of the swine influenza viruses was as high as 6.1. In the BE these differences were decreasing and they were even no longer significant in the LE. Although all AIVs preferentially bind Neu5Acα2-3Galβ-HexNAc-terminated receptors, duck and chicken viruses differ by their recognition of the inner Galβ1-3HexNAc or Galβ1-4HexNAc linkages respectively (Gambaryan et al. 2005). Still we did not observe a clear distinction in virus yield between the examined duck and chicken viruses.

Overall, the differences between the virus yields of swine and AIVs were statistical significant in NE, TE and BE at 24 and 48 hpi and in LE at 24 hpi only. Titers of A/New Caledonia/20/99 (H1N1) were consistently lower than those of swine influenza viruses in NE, TE and BE (except at 24 hpi in the BE). In the same explants the titers of A/Panama/2007/99 (H3N2) were invariably higher than those of AIVs.

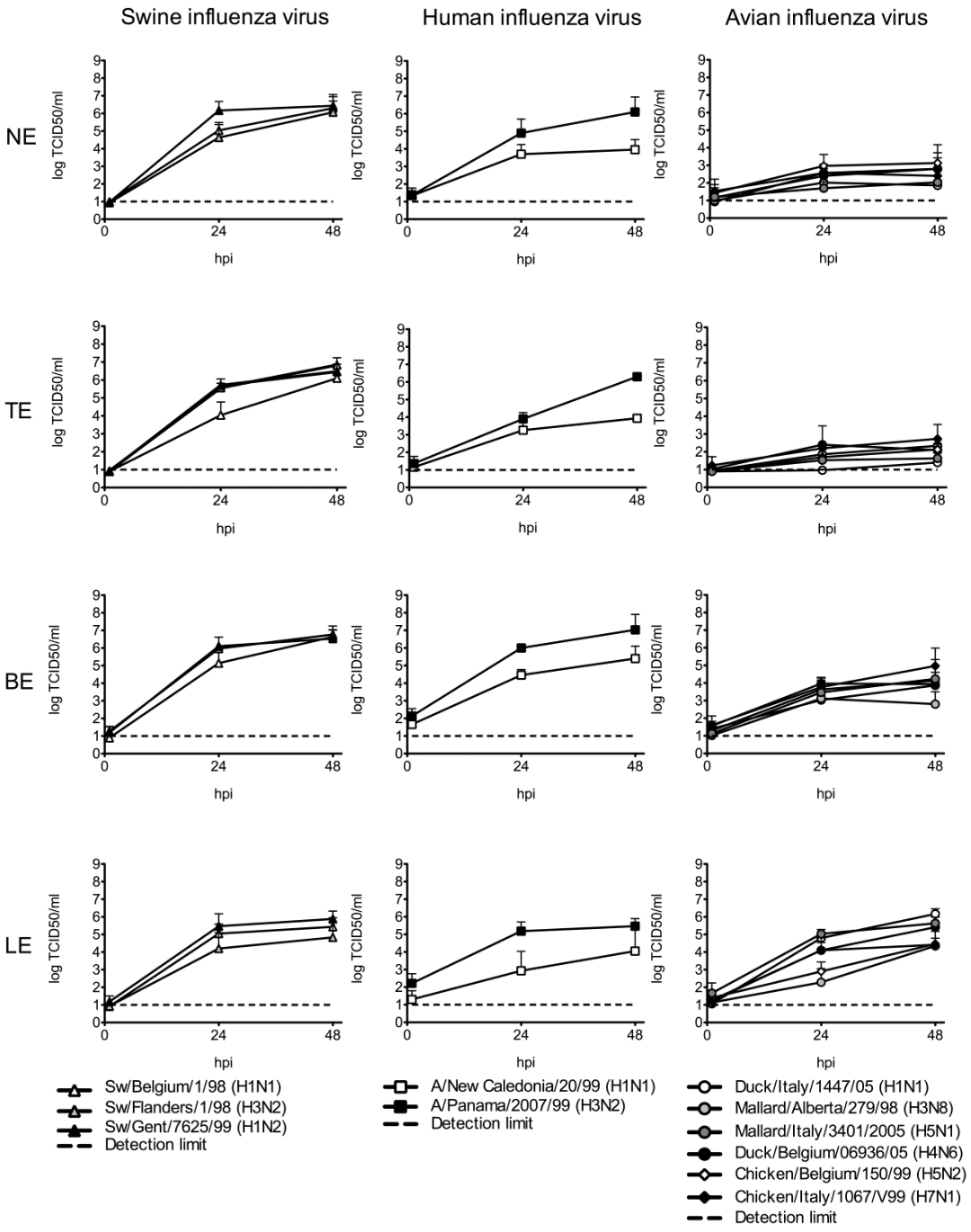

Figure 1. Virus yields, expressed as log $TCID_{50}$/ml, in the supernatant of the explants. Virus titers were determined at 1, 24 and 48 hpi. Each row shows the results per explant system, from NE down to LE. Each column represents the host from which the different virus subtypes were isolated: pigs, humans and birds. Each value is the mean of three experiments, bars show the S.D. NE: nasal explants, TE: tracheal explants, BE: bronchial explants, LE: lung explants

3.4.4. Dose response curves

Figure 2 shows the effect on the virus yield of Swine/Gent/7625/99 (H1N2), Duck/Belgium/06936/05 (H4N6) and Chicken/Belgium/150/99 (H5N2) after inoculation with 10- and 100-fold lower doses (5

and 4 $log_{10}EID_{50}$ respectively) than in the principal experiment. The reduction of the inoculation dose clearly had more effect on the AIVs than on the swine influenza virus. Inoculation of AIVs at 10^4 EID_{50} did not result in infection of the explants (titers below the detection limit), while for swine influenza viruses this was only true for NE and TE. In the BE and LE there was a limited or no reduction of the swine influenza virus yield respectively.

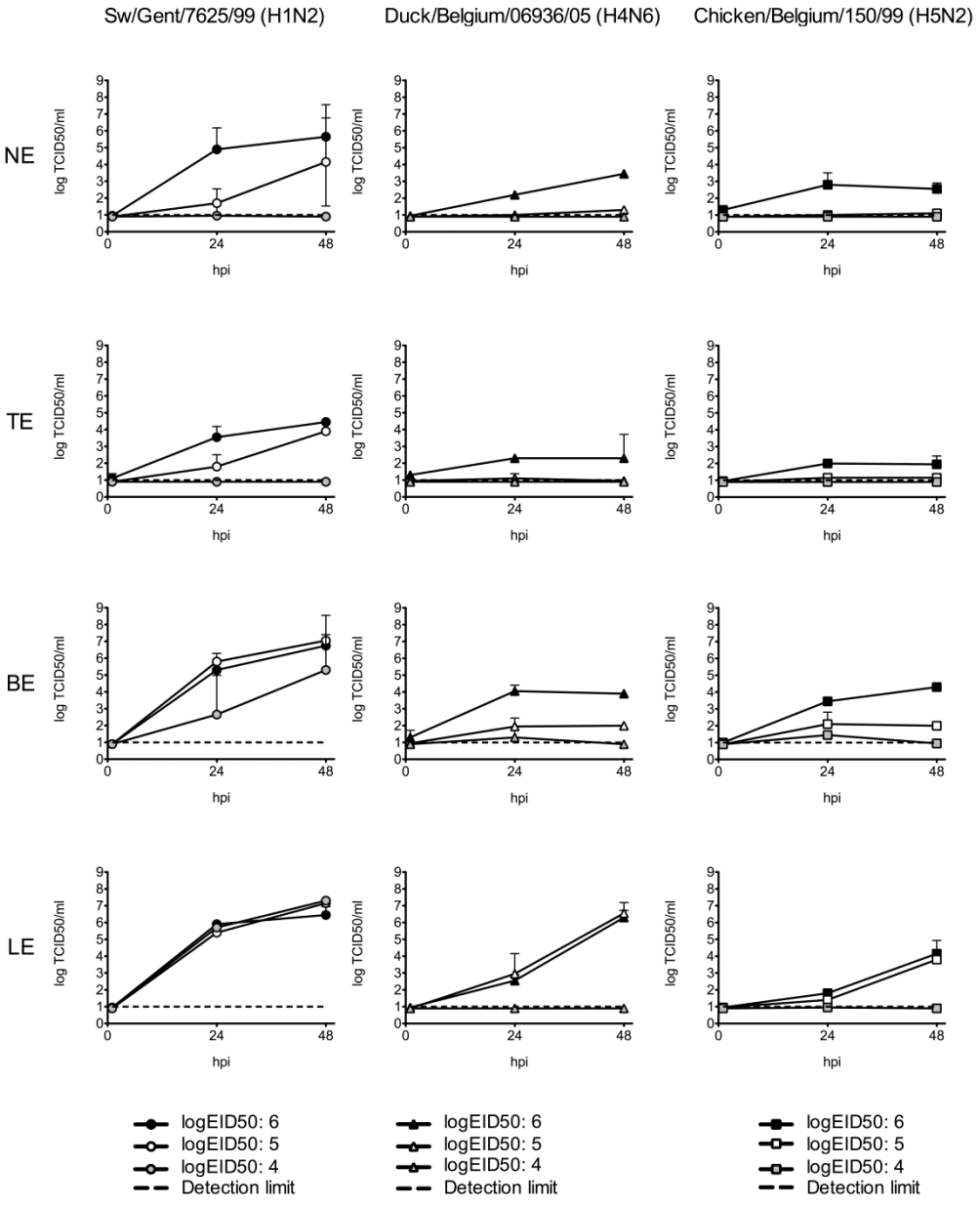

Figure 2. Dose response curves for Sw/Gent/7625/99, Duck/Belgium/06936/05 and Chicken/Belgium/150/99. Three different inoculation doses were applied: 10^6, 10^5 and 10^4 log EID_{50}. Each row represents one explant system, each column one influenza virus. The values are the mean of two experiments, bars show the S.D. NE: nasal explants, TE: tracheal explants, BE: bronchial explants, LE: lung explants

A 10-fold increase of the inoculation dose (10^5 EID_{50}) of AIVs still failed to infect NE or TE. Detectable virus titers were reached in the BE and similar titers as those obtained with the highest inoculation dose in LE. The same dose of swine influenza virus resulted in infection of all explant systems by 48 hpi at levels (almost) identical to the original 10^6 EID_{50} dose. The slope of the virus yields between 1 and 24 hpi was remarkably less steep in NE and TE than for the highest inoculation dose.

3.4.5. Influenza A nucleoprotein detection

An overview of the results is shown in Figure 3. Generally, cells positive by IHC displayed an intense brown intranuclear staining. They were identified in all the explant systems inoculated with the swine influenza virus (H1N2) and only in LE with the AIV (H4N6). Swine influenza virus positive cells in NE and TE were limited to diffusely spread single cells in basal and apical layers of the epithelium with distinctly more positive cells in the NE than in the TE. In the BE the level of infection was higher than in NE and TE, with up to 100% of the epithelium staining positive. Additionally the BE epithelium showed reactive atypia changing to a monolayer with few ciliated cells. Many swine influenza positive cells were also found in the LE. These contained groups of positive epithelial cells or an entirely positive epithelial lining in large and small bronchioles and, rarely, single positive alveolar cells. Detection of AIV positive cells was limited to the bronchioles of LE, with fewer foci and numbers of positive cells than for swine influenza viruses. Semi-quantitative analysis of the IF stainings confirmed these findings, as presented by the symbols in Figure 3.

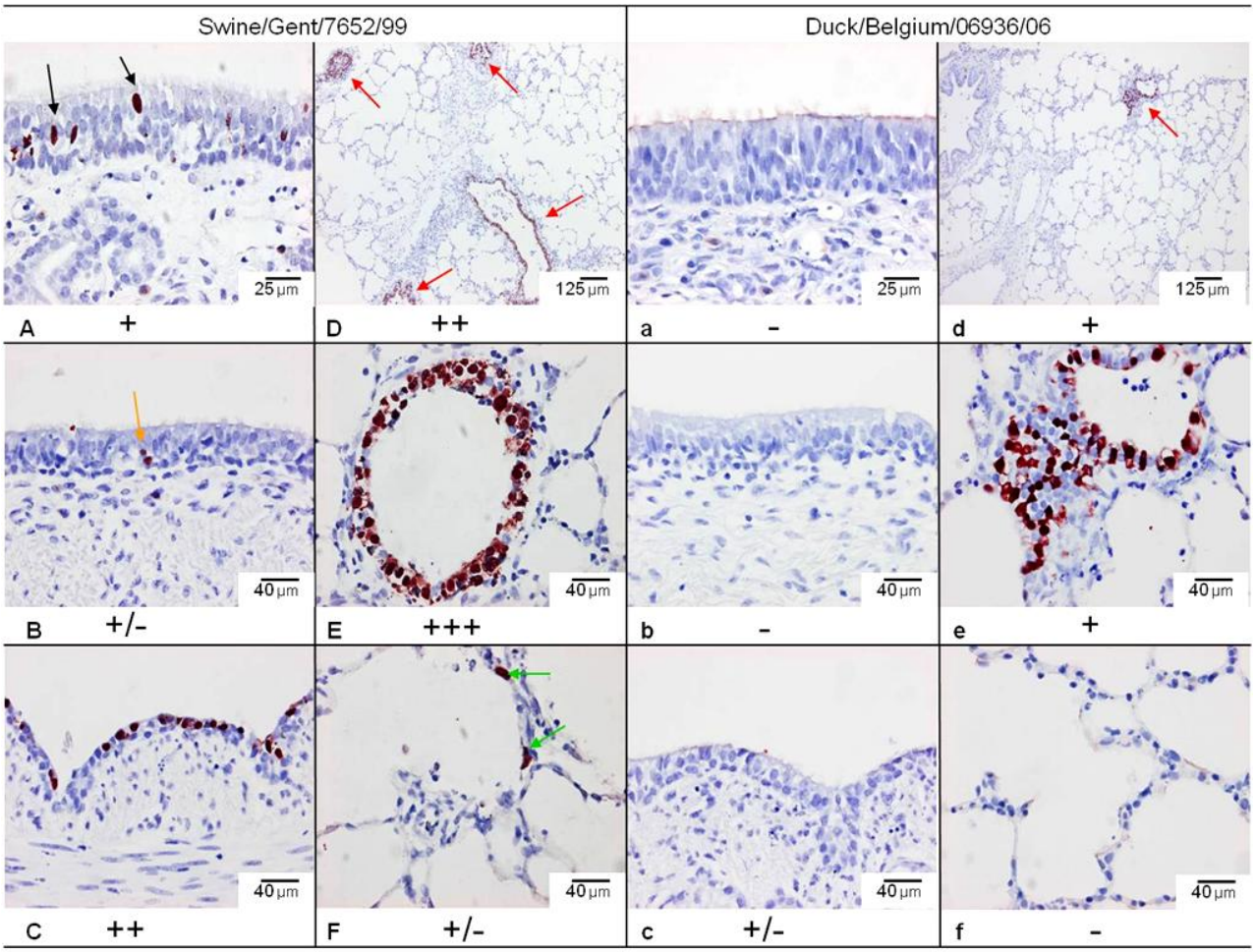

Figure 3. Immunohistochemical analysis of infected cells.

Nasal (A, a), tracheal (B, b), bronchial (C, c) and lung (D→F, d→f) explants at 48 hpi inoculated with Swine/Gent/7625/99 (H1N2) (A→F) and Duck/Belgium/06936/06 (H4N6) (a→f) were analyzed. In the nasal (A: black arrow) and tracheal (B: orange arrow) explants, single swine influenza virus positive cells were diffusely spread while no avian influenza virus positive cells were present (a,b). Swine influenza virus positive cells were also found as a continuous line in bronchial epithelium (C), as multiple foci in the bronchioles (D: red arrows, E) and as single alveolar cells (F: green arrows) in lung explants. Avian influenza viral antigen-positive cells were limited to bronchiolar epithelium in lung explants (d: red arrows, e).

Symbols underneath the pictures give the results for the semi-quantitative analysis of influenza virus positive cells by IF. -: no virus positive epithelial cells, +/-: single positive cells covering <10% of the epithelium, +: between 11 and 40% of the epithelium is positive, ++: between 41 and 70% of the epithelium is positive, +++: between 71 and 100% of the epithelium is positive.

3.4.6. Receptor expression

To determine the Sia receptor distribution in the pig from the nasal mucosa down to the alveoli we performed lectin histochemistry. Considering the results by van Riel et al. (2007) on the pattern of viral attachment (PVA) of human and AIVs in pig tissues, we focused on the expression in epithelial cells and glands of NE, TE and BE and in bronchioles and alveoli of LE. An overview of the results is shown in Table 3.

Both α2-3 and α2-6 linked Sia receptors were detected in the epithelium of the respiratory tract, but they displayed a very distinct distribution pattern. SNA binding (specific toward α2-6Gal linked Sia) was abundant from the nasal epithelium down to the bronchioles, and more moderate in the alveoli (Figure 4). The MAA-I and MAA-II isotypes, which identify Neu5Ac(α2-3)Gal(β1-4)GlcNAc and Neu5Ac(α2-3)Gal(β1-3)GalNAc respectively (Imberty et al. 2000), gave very different results. While MAA-I binding was absent in all epithelial cells, MAA-II binding was rare in nasal, tracheal and bronchial epithelium and moderate in bronchioles and alveoli. At the level of the glands, SNA binding intensity gradually increased from the NE towards the BE. On the contrary, MAA-I and MAA-II were only binding in the glands of NE at a moderate level.

Table 3: Summary of the lectin binding intensities of *Sambucus nigra* agglutinin (SNA) and *Maackia amurensi*s agglutinin I and II (MAA-I and MAA-II) in the porcine respiratory explants.
NE: nasal explants, TE: tracheal explants, BE: bronchial explants, LE: lung explants
-: no binding, +/-: rare binding, +: moderate binding, ++: abundant binding

		SNA	MAA-I	MAA-II
NE	Epithelium	++	-	+/-
	Glands	+/-	+	+
TE	Epithelium	++	-	+/-
	Glands	+	+/-	-
BE	Epithelium	++	-	+/-
	Glands	++	+/-	-
LE	Bronchioles	++	-	+
	Alveoli	+	-	+

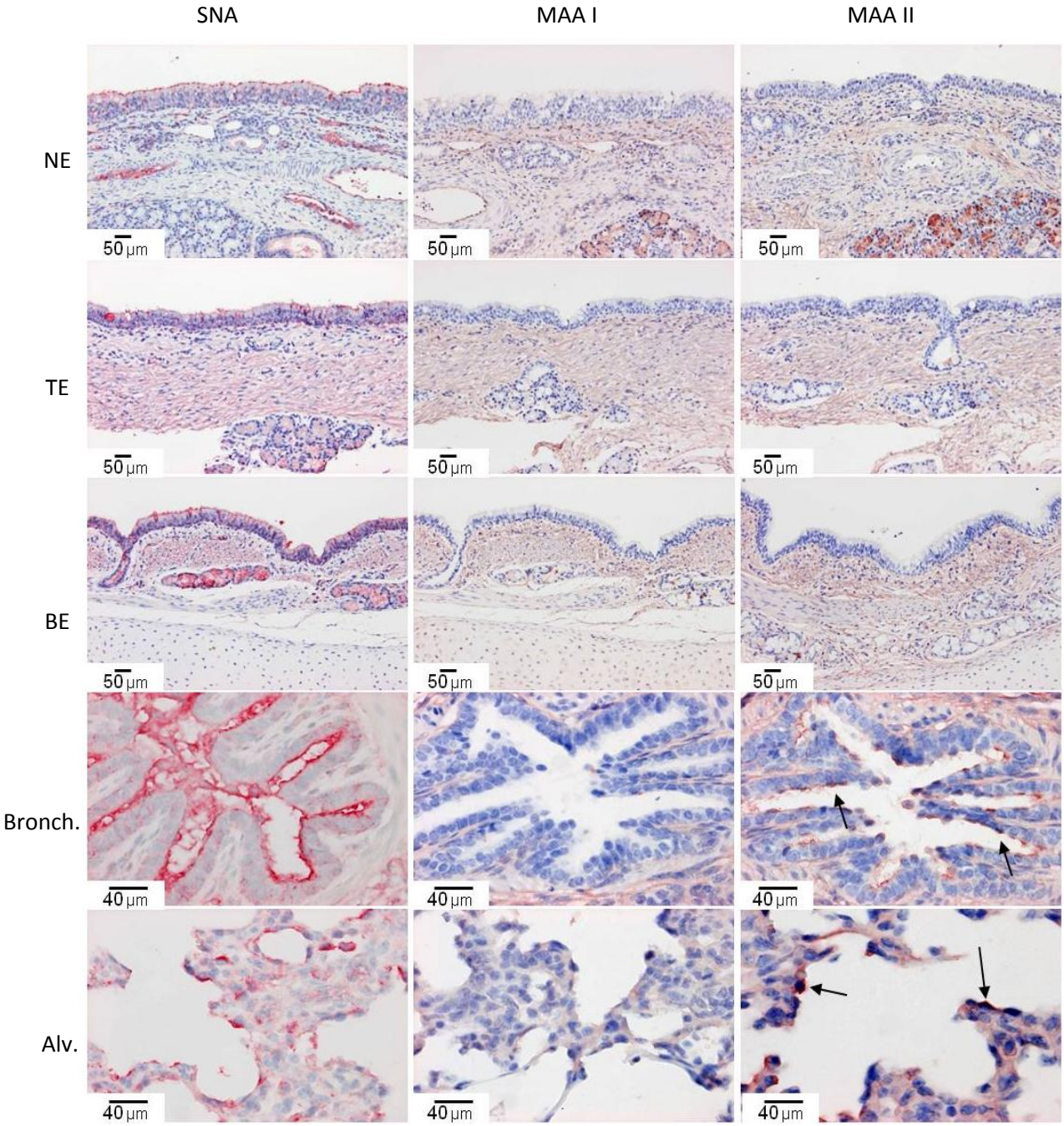

Figure 4. Tissue binding of Sambucus nigra agglutinin (SNA), Maackia amurensis agglutinin I (MAA-I) and Maackia amurensis agglutinin II (MAA-II) in the different explant systems. SNA binding (first column) was abundant in the epithelium of nasal (NE), tracheal (TE) and bronchial explants (BE) and in the epithelium of bronchioles (Bronch.), but moderate at the level of the alveoli (Alv.). MAA-I binding to epithelial cells was absent to rare in all explants systems (second column). MAA-II binding (third column) was rare in the epithelium of NE, TE and BE. At the level of the bronchioles and the alveolar tissue, it became moderate to abundant (as indicated by the black arrows).

Since our findings of lack of binding with MAA-I and -II in the trachea were in disagreement with previous reports of Ito et al. (1998) and Suzuki et al. (2000), we tried to find an explanation for the discrepant results. Both used acetone fixed tracheal cryosections and digoxigenin labeled MAA (Dig-MAA). Duck intestines were used as a positive control. Therefore, we compared Dig-MAA binding on

acetone fixed cryosections of the trachea with that on paraffin sections of paraformaldehyde fixed tissues. The frozen tissues still showed no binding of MAA to the tracheal epithelium but more positive binding to the submucous glands and to blood vessels (Figure 5).

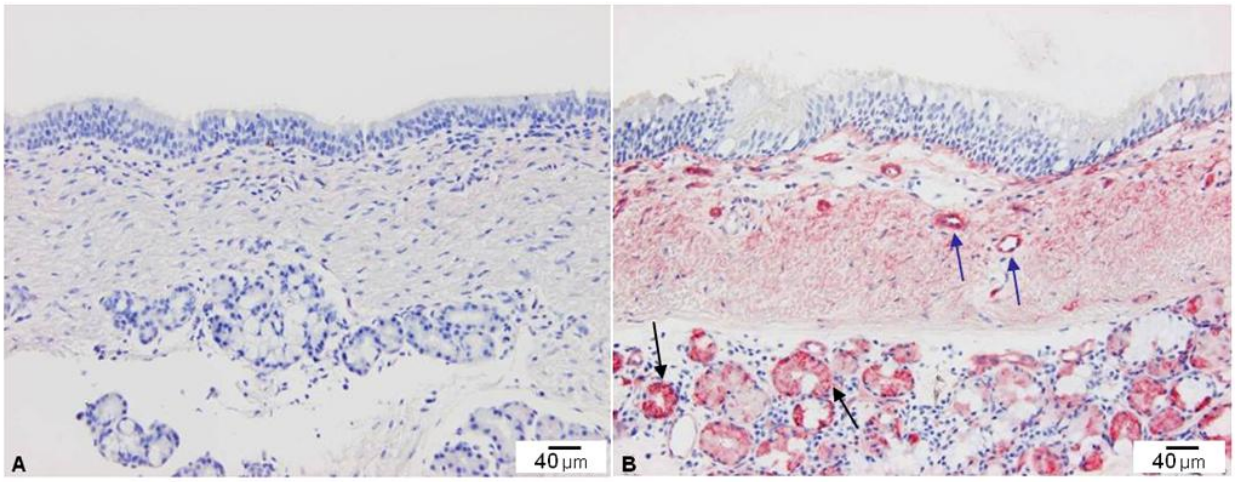

Figure 5. Comparison of binding with digoxigenin-conjugated MAA in paraffin sections (A) and cryosections (B) of the porcine trachea. Only the cryosections showed clear positivity in the glands (black arrows) and the small blood vessels (blue arrows), while paraffin sections were completely negative.

Because our MAA lectins were biotinylated instead of digoxigenin labeled we also wanted to exclude that the different conjugation method was the cause of the negative binding in the porcine trachea. For that reason we compared the binding of biotinylated MAA-I and –II with digoxigenin labeled MAA-I and –II in duck intestines. This tissue is traditionally used as a positive control because it only expresses Siaα2-3Gal linkages. The digoxigenin labeled MAA-I and II, as shown in Figure 6 panel C and c respectively, gave no binding. The biotinylated MAA-I and –II were both binding to the intestinal epithelium but in a different pattern. The MAA-I (panel A) bound only to the apical surface of the epithelium, while MAA-II (panel a) also bound to the mucus of the goblet cells. The binding was shown to be specific, since it was abolished when the sections were pretreated with neuraminidase (panels B and b). In the porcine trachea there was no binding of either biotinylated nor digoxigenin labeled MAA.

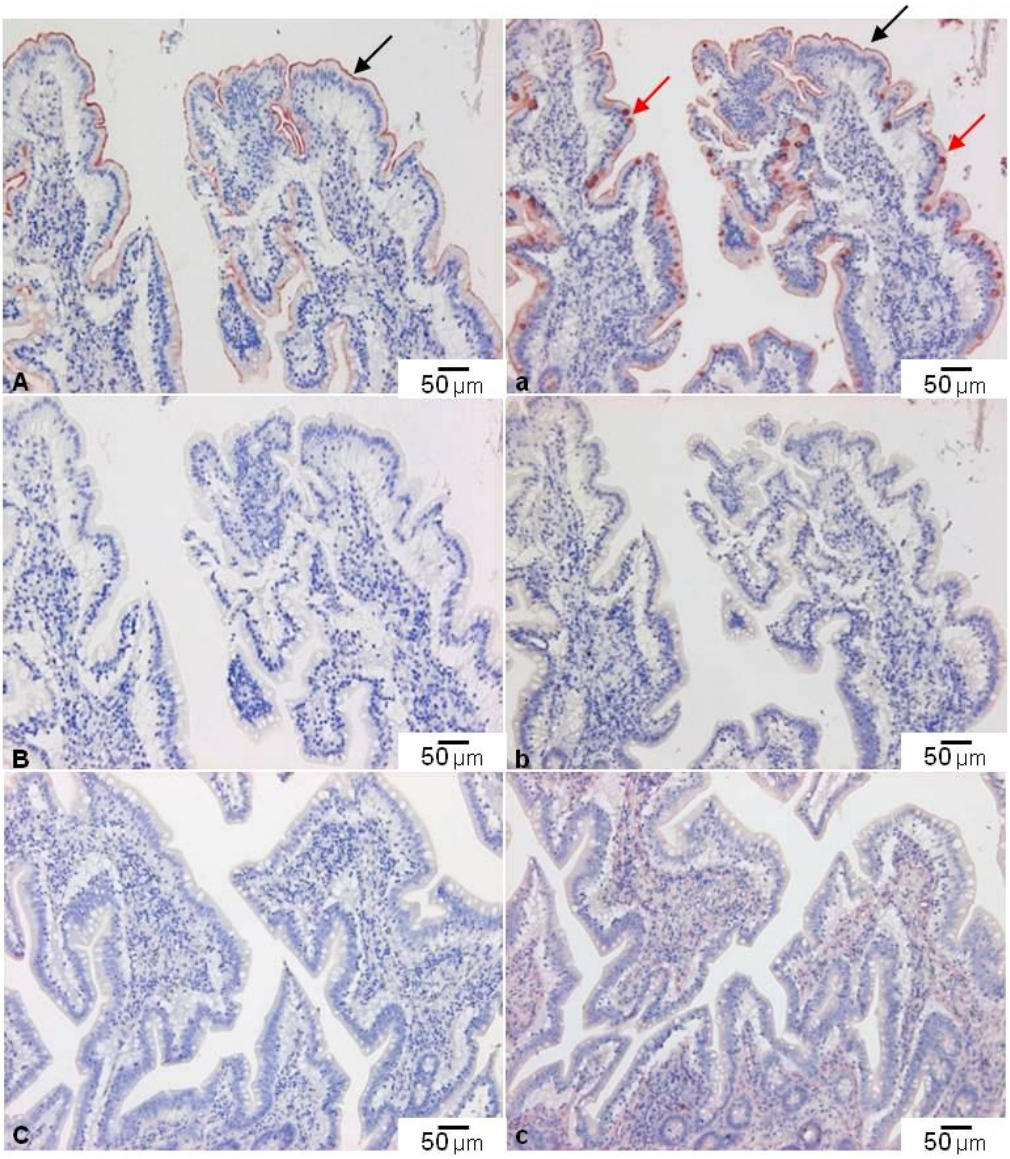

Figure 6. Influence of the conjugation method of MAA-I and -II lectins on the staining intensities in duck small intestines. Biotinylated MAA-I (A) and MAA-II (a) both resulted in epithelial cell binding (black arrows), but MAA-II (a) was additionally staining the goblet cells (red arrow). For both lectins binding was abolished by sialidase treatment of the sections (B,b). Digoxigenin labelled MAA-I (C) and MAA-II (c) failed to bind to the same tissues.

3.5. Discussion

We have confirmed the susceptibility of porcine respiratory tissues to infection with a range of AIVs. These AIVs replicated clearly less efficiently in tissues of the upper (nasal and tracheal) than in the lower (bronchi and alveolar) respiratory tract. This was associated with a paucity of α2-3 linked Sia receptors in the nose and trachea.

The relatively low AIV titers in porcine NE and TE may in part explain why experimental pig-to-pig transmissions of AIVs have failed so far (Loeffen et al. 2004, De Vleeschauwer et al. 2009). This hypothesis is further strengthened by the results of our dose response experiments, in which a 10-fold reduction of the inoculation dose of AIVs completely abolished infection in NE and TE. A similar

10-fold reduction of the inoculation dose of a swine influenza viruses did not eliminate infection, indicating that the predominant distribution of an appropriate receptor is indeed an important determinant for cell tropism (Kuiken et al. 2006). Wild birds infected with low pathogenic AIVs mainly excrete the virus via fecal and oculonasal discharges, while aerosol transmission is much less important (Boyce et al. 2009). We therefore speculate that a successful infection of the porcine upper respiratory tract (URT) with AIVs requires exposure to feces or fecal contaminated material with high virus concentrations. However, the likelihood that an entirely AIV successively infects several pigs, allowing a gradual adaptation to a mammalian host by point mutations, was probably overestimated in the past.

Since the infectivity pattern in our *ex vivo* system is consistent with previous studies on avian, human and swine influenza virus attachment and replication, it is a valuable alternative to *in vivo* experiments. Two recent pig infection studies (Lipatov et al. 2008, De Vleeschauwer et al. 2009) clearly showed a lower replication efficiency for AIVs than for swine influenza viruses throughout the porcine respiratory tract. In both studies the AIVs replicated better in the lower (LRT) than in the upper respiratory tract (URT), but this was also the case for the swine influenza viruses. The latter is not always consistent with findings in our *ex vivo* system, in which swine influenza viruses sometimes reached lower titers in LEs than in NEs. When comparing the virus titers of a single virus obtained in the 4 different systems we need to keep in mind that these explants, although standardized in size, do not automatically contain an identical number of virus susceptible cells.

Our results on lectin binding intensities were not entirely in line with previous studies. We confirmed the abundant expression of α2-6 linked Sia receptors in the trachea as well as in other parts of the respiratory tract, but α2-3 linked Sia receptors were only detected in the bronchioles and alveoli, with moderate intensity. Overall we showed the Sia receptor distribution in the pig tissues to be similar to that in humans (Shinya et al. 2006, Nicholls et al. 2007, Yao et al. 2008). Even when repeating the methods of Ito et al. (1998), no α2-3 linked Sia receptors could be identified in the trachea. Van Riel et al. (2007) have previously studied the pattern of virus attachment in porcine respiratory tissues using labeled avian and human influenza viruses. Human viruses attached to many cells in the trachea, bronchus, bronchioles and to a moderate number in the alveoli, which is in agreement with our SNA binding intensities. As for the avian viruses, there was a lack of binding in trachea and bronchus, but increased binding in the lung, which is in accordance with our MAA-II staining. These patterns of viral attachment therefore agree with our lectin stainings, and they dispute the much cited study by Ito et al. It is of interest to note that chicken and duck influenza

isolates are known to prefer SAα2-3Galβ1-4GlcNAc (as recognized by MAA-I) and SAα2-3Galβ1-3GalNAc (as recognized by MAA-II) respectively (Yamamoto et al. 1997, Gambaryan et al. 2005). As MAA-I binding in all the explant systems was negative, we would expect a reduced replication potential of the chicken isolates, which was not the case. This contradicting result could be explained by the hypothesis of Guo et al. (2007), who state that Sia are necessary but not sufficient to act as the cellular receptor. If an additional internalizing receptor is involved in influenza infection, this could account for the examples where influenza virus entry did not seem to be affected by a depletion of cell surface Sia (Thompson et al. 2006, Glaser et al. 2007).

Even within one group of heterologous viruses, some possess a higher infectivity than others. A/New Caledonia/20/99 (H1N1) had a 2 $\log_{10}$ lower viral yield than A/Panama/2007/99 (H3N2). Though both viruses are expected to have mainly a Sia α2-6 receptor specificity Wan and Perez (2006) and Glaser et al. (2007), have suggested a dual receptor specificity (for both human- and avian-like receptors) for A/New Caledonia/20/99 (H1N1) and a strict Sia α2-6 preference for A/Panama/2007/99 (H3N2). To assess whether certain viruses are more likely to undergo interspecies transmissions, molecular distinctions responsible for this difference in infectivity will have to be identified.

In this study we successfully developed an *ex vivo* model that covers the entire porcine respiratory tract and is permissive to influenza virus replication in a similar way as *in vivo*. The infectivity of AIVs was shown to be low in the URT, while the replication capacity of human influenza viruses more closely resembled that of swine influenza viruses. These findings correlated with the Sia receptor distribution in the pig tissues, which was shown to be similar to that in humans. Consequently, the classical hypothesis on the unique role of the pig as a mixing vessel, based on the abundant expression of both α2-3 linked and α2-6 linked Sia receptors in the trachea, no longer stands. Simultaneous presence of human- and avian-type receptors has also been identified in humans (Shinya et al. 2006, Nicholls et al. 2007, Yao et al. 2008), ducks and quail (Wan and Perez 2006, Kuchipudi et al. 2009). Additionally, Thompson and colleagues (2006) have generated data indicating that co-infection of human ciliated epithelial cells with human and avian influenza viruses could occur. Therefore, more detailed studies on the mechanism and on predisposing factors of reassortment are required to assess the true role of the pig.

3.6. Acknowledgements

This work was supported by the EU funded project FLUPATH (contract CT044220). Special thanks go to Lieve Sys, Kevin Fung, Nele Dennequin, Melanie Bauwens and Geert Opsomer for excellent

technical support and to Ilaria Capua, Alan Hay, Thierry van den Berg and Robert Webster for providing the viruses. S. Van Poucke would also like to express her gratitude to M. Matrosovich, J. Uhlendorff and S. Haslam for inspirational discussions.

3.7. References

Boyce W.M., Sandrock C., Kreuder-Johnson D. et al. (2009). Avian influenza viruses in wild birds: a moving target. Comp Immunol Microbiol Infect Dis 32: 275-286.

Brinkman-Van der Linden E.C.M., Sonnenburg J.L., Varki A. (2002). Effects of sialic acid substitutions on recognition by Sambucus nigra agglutinin and Maackia amurensis hemagglutinin. Anal Biochem 303: 98-104.

Brown I.H., Harris P.A., McCauley J.W. et al. (1998). Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. J Gen Virol 79: 2947-2955.

Brown I.H. (2000). The epidemiology and evolution of influenza viruses in pigs. Vet Microbiol 74: 29-46.

Busch M.G., Bateman A.C., Landolt G.A. et al. (2008). Identification of amino acids in the HA of H3 influenza viruses that determine infectivity levels in primary swine respiratory epithelial cells. Virus Res 133: 269-279.

Castrucci M.R., Donatelli I., Sidoli L. et al. (1993). Genetic Reassortment between Avian and Human Influenza A Viruses in Italian Pigs. Virology 193: 503-506.

Choi Y.K., Nguyen T.D., Ozaki H. et al. (2005). Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol 79: 10821-10825.

Darzynkiewicz Z., Juan G., Li X. et al. (1997). Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). Cytometry 27: 1-20.

De Vleeschauwer A., Atanasova K., Van Borm S. et al. (2009a). Comparative Pathogenesis of an Avian H5N2 and a Swine H1N1 Influenza Virus in Pigs. PLoS ONE 4: doi:10.1371/journal.pone.0006662.

De Vleeschauwer A., Van Poucke S., Braeckmans D. et al. (2009b). Efficient transmission of swine-adapted but not wholly avian influenza viruses between pigs and from pigs to ferrets. J Infect Dis 200: 1884-1892.

Gambaryan A., Yamnikova S., Lvov D. et al. (2005). Receptor specificity of influenza viruses from birds and mammals: New data on involvement of the inner fragments of the carbohydrate chain. Virology 334: 276-283

Glaser L., Conenello G., Paulson J. et al. (2007). Effective replication of human influenza viruses in mice lacking a major α2,6 sialyltransferase. Virus Res 126: 9-18.

Glorieux S., Van den Broeck W., Van der Meulen K.M. et al. (2007). In vitro culture of porcine respiratory nasal mucosa explants for studying the interaction of porcine viruses with the respiratory tract. J Virol Methods 142: 105-112.

Guan Y., Shortridge K.F., Krauss S. et al. (1996). Emergence of avian H1N1 influenza viruses in pigs in China. J Virol 70: 8041-8046.

Guo Y., Rumschlag-Booms E., Wang J. et al. (2009). Analysis of hemagglutinin-mediated entry tropism of H5N1 avian influenza. Virol J 6: doi: 10.1186/1743-422X-6-39.

Hinshaw V.S., Webster R.G., Easterday B.C. et al. (1998). Replication of Avian Influenza A Viruses in Mammals. Infect Immun 34: 354-361.

Imberty A., Gautier C., Lescar J. et al. (2000). An unusual carbohydrate binding site revealed by the structures of two Maackia amurensis lectins complexed with sialic acid-containing oligosaccharides. J Biol Chem 275: 17541-17548.

Ito T., Couceiro J., Kelm S et al. (1998). Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol 72: 7367-7373.

Karasin A.I., Brown I.H., Carman S. et al. (2000a). Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. J Virol 74: 9322-9327.

Karasin A.I., Schutten M.M., Cooper L.A. et al. (2000b). Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977-1999: evidence for wholly human and reassortant virus genotypes. Virus Res 68: 71-85.

Karasin A.I., West K., Carman S. et al. (2004). Characterization of avian H3N3 and H1N1 influenza A viruses isolated from pigs in Canada. J Clin Microbiol 42: 4349-4354.

Kida H., Ito T., Yasuda J. et al. (1994). Potential for transmission of AIVs to pigs. J Gen Virol 75: 2183-2188

Klenk H., Matrosovich M., Stech J. (2008). Avian Influenza: Molecular Mechanisms of Pathogenesis and Host Range. In Animal Viruses Molecular Biology. Edited by Mettenleiter TC. Norfolk: Caister Academic Press;253-303.

Kuchipudi S.V., Nelli R., White G.A. et al. (2009). Differences in influenza virus receptors in chickens and ducks: implications for interspecies transmission. J Mol Genet Med 3: 143-151.

Kuiken T., Holmes E.C., McCauley J. et al. (2006). Host species barriers to influenza virus infections. Science 312: 394-397.

Landolt G.A., Karasin A.I., Phillips L. et al. (2003). Comparison of the pathogenesis of two genetically different H3N2 influenza a viruses in pigs. J Clin Microbiol 41: 1936-1941.

Lipatov A.S., Kwon Y.K., Sarmento L.V. et al. (2008). Domestic pigs have low susceptibility to H5N1 highly pathogenic avian influenza viruses. PloS Pathogens 4:.doi: 10.1371/journal.ppat.1000102

Loeffen W., de Boer E., Koch G. (2004). Transmission of a highly pathogenic avian influenza virus to swine in the Netherlands [abstract]. In-between congress of the International Society for Animal Hygiene 2004, 329-330.

Ma W, Kahn R.E., Richt J.A. (2009). The pig as a mixing vessel for influenza viruses: Human and veterinary implications. J Mol Genet Med 3: 158-166.

Nicholls J.M., Bourne A.J., Chen H. et al. (2007a). Sialic acid receptor detection in the human respiratory tract: evidence for widespread distribution of potential binding sites for human and avian influenza viruses. Resp Res 8. doi: 10.1186/1456-9921-8-37.

Nicholls J.M., Chan M.C.W., Chan W.Y. et al. (2007b). Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nat Med 13: 147-140.

Ninomiya A., Takada A., Okazaki K. et al. (2002). Seroepidemiological evidence of avian H4, H5, and H9 influenza A virus transmission to pigs in southeastern China. Vet Microbiol 88: 107-114.

Ohuchi M., Ohuchi R., Feldmann A. et al. (1997). Regulation of receptor binding affinity of influenza virus hemagglutinin by its carbohydrate moiety. J Virol 71: 8377-8384.

Olsen C.W., Brown I.H., Easterday B.C. et al. (2006). Swine Influenza. In Diseases of Swine. 9th edition. Edited by Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ. Ames: Iowa State University Press: 469-482.

Peiris J.S.M., Guan Y., Markwell D. et al. (2001). Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern China: Potential for genetic reassortment? J Virol 75: 9679-9686.

Porro G.A., Lee J.H., De Azavedo J. et al. (2001). Direct and indirect bacterial killing functions of neutrophil defensins in lung explants. Am J Physiol Lung Cell Mol Physiol 281: L1240-L1247.

Schmidt R.C., Maassab H.F., Davenport F.M. (1974). Infection by Influenza A Viruses of Tracheal Organ Cultures Derived from Homologous and Heterologous Hosts. J Infect Dis 129: 28-36.

Scholitssek C. (1990). Pigs as the 'mixing vessel' for the creation of new pandemic influenza A viruses. Med Princip Prac 2: 65-71.

Shinya K., Ebina M., Yamada S. et al. (2006). Influenza virus receptors in the human airway. Nature 440: doi: 10.1038/440435a.

Shortridge K.F., Zhou N.N., Guan Y., et al. (1998). Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 252: 331-342.

Subbarao K., Swayne D.E., Olsen C.W. (2006). Epidemiology and control of human and animal influenza. In Influenza Virology Current Topics. Edited by Kawaoka Y. Wyomandham: Caister Academic Press;229-280.

Suzuki Y., Ito T., Suzuki T. et al. (2000). Sialic acid species as a determinant of the host range of influenza A viruses. J Virol 74: 11825-11831.

Thompson C.I., Barclay W.S., Zambon M.C. et. al. (2006). Infection of human airway epithelium by human and avian strains of influenza A virus. J Virol 80: 8060-8068.

van Riel D., Munster V.J., de Wit E. et al. (2007). Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am J Pathol 171: 1215-1223.

Wan H.Q. and Perez D.R. (2006). Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. Virology 346: 278-286.

Yamamoto K., Konami Y., Irimura T. (1997). Sialic acid-binding motif of Maackia amurensis lectins. J Biochem, 121:756-61.

Yao L., Korteweg C., Hsueh W. et al. (2008). Avian influenza receptor expression in H5N1-infected and noninfected human tissues. FASEB J 22: 733-740.

Zhou N.N., Senne D.A., Landgraf J.S. et al. (1999). Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol 73: 8851-8856.

CHAPTER 4.

HOW A SWITCH IN RECEPTOR SPECIFICITY AFFECTS REPLICATION, TRANSMISSION AND CELL TROPISM OF A/HONG KONG/1/68 (H3N2) IN PIGS

4.1.

Effect of receptor specificity of A/Hong Kong/1/68 (H3N2) influenza virus variants on replication and transmission in pigs

Van Poucke S., Uhlendorff J., Wang Z., Billiau V., Nicholls J., Matrosovich M., Van Reeth K.

Influenza and Other Respiratory Viruses (2012), doi: 10.1111/j.1750-2659.2012.00376

4.1.1. Abstract

Several arguments plead for an important role of pigs in human influenza ecology, including the similar receptor expression pattern in the respiratory tract of both species. How virus receptor binding specificity affects transmission in pigs, on the other hand, has not been studied so far.

Using recombinant virus R1-HK which harbored all genes from the original pandemic virus A/Hong Kong/1/68 (H3N2) and R2-HK which differed by L226Q and S228G mutations in the hemagglutinin and conversion to an avian-virus-like receptor specificity, we assessed the role of receptor specificity on (1) replication in porcine respiratory explants, (2) pig-to-pig transmission and (3) replication and organ tropism in pigs.

In nasal, tracheal and bronchial explants we noticed a 10- to 100-fold lower replication of R2-HK compared to R1-HK. In the lung explants, the viruses replicated with comparable efficiency. These observations correlated with the known expression level of Siaα2-3Gal in these tissues. In the pathogenesis study, virus titers in the respiratory part of the nasal mucosa, the trachea and the bronchus were in line with the ex vivo results. R2-HK replicated less efficiently in the lungs of pigs than R1-HK, which contrasted with the explants results. R2-HK also showed a pronounced tropism for the olfactory part of the nasal mucosa. Transmissibility experiments revealed that pig-to-pig transmission was abrogated when the virus obtained Siaα2-3Gal binding preference. Our data suggest that Siaα2-6Gal binding is required for efficient transmission in pigs.

4.1.2. Introduction

Introduction of a new influenza virus to which the human population has no or little pre-existing immunity and that is capable of transmitting from humans to humans, poses a pandemic threat. Such a virus can arise from genetic reassortment between avian and mammalian influenza viruses or by the adaptation of a non-human influenza virus (Kilbourne 2006). Although aquatic birds are the major influenza virus reservoir, pigs are supposed to play an important role in human influenza ecology. Swine have been postulated to be either an intermediate host for the transmission of avian influenza viruses (AIVs) to humans or a mixing vessel for avian and mammalian viruses (Scholtissek et al. 1985, Claas et al. 1994, Ma et al. 2009). This is based on the observation that most, if not all, avian virus subtypes can replicate in swine (Kida et al. 1994, De Vleeschauwer et al. 2009). Furthermore, both terminal sialic acid (Sia)α2-3Gal and Siaα2-6Gal, the preferred virus receptors of avian and human influenza viruses respectively, were shown to be present in the porcine trachea (Ito et al. 1998). More recent research has confirmed the abundant expression of Siaα2-6Gal on the epithelial lining of the porcine respiratory tract, but Siaα2-3Gal was detected only sparsely in the upper respiratory tract (Nelli et al. 2010, Van Poucke et al. 2010, Trebbien et al. 2011). Hence, the receptor distribution pattern in the respiratory tract of pigs shows extensive similarity with that of humans (Shinya et al. 2006, Yao et al. 2008). More detailed pathogenesis studies have shown that although pigs support replication of many AIVs, the titers reached in the respiratory tract are modest (Shortridge et al. 1998, Choi et al. 2005, Lipatov et al. 2008, De Vleeschauwer et al. 2009). Also, low and highly pathogenic AIVs could not transmit from pig to pig, while swine adapted viruses could under the same experimental conditions (Shortridge et al. 1998, Choi et al. 2005, De Vleeschauwer et al. 2009).

A large body of data highlights the importance of the viral hemagglutinin (HA) glycoprotein for the host range restriction. Thus, the pandemic “Hong Kong” H3N2 influenza virus emerged by reassortment of the then circulating human H2N2 virus with an avian H3 influenza virus and the avian HA showed a switch in receptor binding specificity shortly after the introduction of the virus in the human population in 1968 (Connor et al. 1994, Matrosovich et al. 2000).

Residues 226 and 228 in the HA of H3N2 influenza virus strains have been shown to determine receptor binding specificity. When the HA harbors leucine at 226 and serine at 228, the virus binds Siaα2-6Gal (human-type receptor), whereas glutamic acid at 226 and glycine at 228 preferentially recognize Siaα2-3Gal (avian-type receptor) (Vines et al. 1998, Matrosovich et al. 2007). In cultures of human tracheobronchial epithelial cells, recombinant viruses with these mutations displayed differences in cell tropism, similar to the ones observed with wild type avian and human influenza viruses (Matrosovich et al. 2004, Scull et al. 2009). Roberts and colleagues used a pair of recombinant

influenza viruses with L226Q/S228G substitutions in the A/Victoria/3/75 (H3N2) backbone to examine their transmissibility in ferrets. They observed a lack of transmission of the mutant virus with avian-virus-like receptor specificity (Roberts et al. 2011).

Global surveillance of influenza viruses in bird, swine and human populations is considered a key factor in the early detection of viruses with pandemic potential. A lack of detained knowledge on molecular determinants of viral host range restriction, especially in pigs, hampers predictions of the pandemic potential of emerging influenza viruses as illustrated by the 2009 pandemic (Garten et al. 2009). Therefore we aimed to determine whether L226Q/S228G mutations in the HA of A/Hong Kong/1/68 (H3N2) would affect (1) replication potential in porcine respiratory explants, (2) replication potential and organ tropism in pigs and (3) pig-to-pig transmission.

4.1.3. Methods

4.1.3.1. Animals

Six- to 8-week-old pigs were obtained from a commercial herd serologically negative for influenza. All animals were tested for influenza virus antibodies before the start of the experiment by hemagglutination inhibition (HI) assay, by a competitive anti-influenza A nucleoprotein enzyme immunoassay (Idexx Laboratories) and by immunoperoxidase monolayer assay (IPMA). Swine/Belgium/1/98 (H1N1), swine/Flanders/1/98 (H3N2) and swine/Gent/7625/99 (H1N2) were used in the HI and IPMA assay. Pigs were housed in separate high-efficiency particulate air-filtered isolation units. At arrival they were treated intramuscularly with ceftiofur (Naxcel®, Pfizer-1ml/20 kg body weight). All experiments were authorized by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine, Ghent University.

4.1.3.2. Viruses

The recombinant viruses R1-HK and R2-HK have been described previously (Matrosovich et al. 2007). They were generated using the eight plasmids reverse genetic system described by Hoffmann et al. (2000) and site-directed mutagenesis to introduce the amino acid substitutions. R1-HK harbors all eight original genes of the pandemic human virus A/Hong Kong/1/68 (H3N2). R2-HK differs from R1-HK solely by the L226Q/S228G mutations in the HA, which lead to a switch in receptor specificity from preferential binding of Siaα2-6Gal (R1-HK) to preferential recognition of Siaα2-3Gal (R2-HK) (Matrosovich et al. 2007). Virus stocks were prepared in MDCK cells and their infectivity was determined by plaque titration in MDCK cells. The low pathogenic AIV duck/Italy/3139-1/06 (H3N2) was provided by Dr. I. Capua. Swine/Flanders/1/98 represents a European human-like H3N2 swine influenza virus. The HA and NA genes of the swine H3N2 virus lineage are derived from descendants

of A/Hong Kong/1/68 and the six gene fragments coding for internal and non-structural proteins from the avian-like H1N1. The amino acids at locations 226 and 228 are identical to those of R1-HK.

4.1.3.3. Porcine respiratory explants systems

Porcine nasal (NE), tracheal (TE), bronchial (BE) and lung (LE) explants were prepared as described elsewhere (Van Poucke et al. 2010). The replication potential of R1-HK, R2-HK, duck/Italy/3139-1/06 (dk/It/06) and swine/Flanders/1/98 (sw/Fl/1/98) inoculated at three different doses (10^4, 10^5 and 10^6 plaque forming units (PFU)) was assessed by determining the virus titers in supernatant at 1, 24 and 48 hours post inoculation. Virus titrations were performed on MDCK cells in 96-well plates followed by an immunocytochemical staining with monoclonal antibody HB-65 against viral nucleoprotein and virus titers were calculated by the method of Reed and Münch (1938).

4.1.3.4. Transmission studies

For each of the recombinant viruses, we used 6 directly inoculated (donor) pigs and 6 contact pigs. The 6 donor pigs of each group were housed in a separate isolation unit and inoculated intranasally with 10^6 PFU of R1-HK or R2-HK on day 0, as described earlier (De Vleeschauwer et al. 2009). Two days after primary inoculation, 6 contact pigs were introduced into each group. The housing allowed both direct and airborne contact between inoculated and contact animals. Virus shedding was monitored by collecting nasal swabs from 0 through 9 days post inoculation (pi) or post contact (pc). Nasal swabs were put into 1 ml of transport medium and used for virus titration in MDCK cells. Blood samples for serological examination were collected at 14 and 28 dpi/dpc. In the group of R2-HK contact pigs, blood was collected at 17 instead of 28 dpc. Transmission was defined by detection of virus from the nasal swabs and/or seroconversion in contact animals.

4.1.3.5. Pathogenesis study

Two separate groups, of 5 pigs each, were inoculated intranasally with 10^6 PFU of R1-HK or R2-HK as described for the transmission study. From 1 until 5 dpi, one pig from each group was euthanized. The following samples were collected for virus titration: nasal mucosa respiratory part, nasal mucosa olfactory part, tonsils, trachea, bronchus (at the level of the bifurcation), bulbus olfactorius and lung. Five different areas of the lung were collected and titrated separately: 1) apical + cardiac lobe right 2) apical + cardiac lobe left 3) diaphragmatic lobe right 4) diaphragmatic lobe left 5) accessory lung lobe. All tissue samples for virus titration were weighed and put in phosphate-buffered saline containing 10 IU /ml penicillin and 10 µg/ml streptomycin to prepare 20% tissue homogenates.

4.1.3.6. Serological examination

Antibody titers against the homologous viruses were determined by hemagglutination inhibition (HI), virus neutralisation (VN) and immunoperoxidase monolayer assay (IPMA). Antibodies against the viral NP were detected by a competitive anti-influenza A nucleoprotein enzyme immunoassay (NP-ELISA). HI and VN assays were performed as described earlier (De Vleeschauwer et al 2010). Sera from the R2-HK group were tested in parallel HI assays with chicken and horse erythrocytes, while sera from R1-HK were tested with chicken erythrocytes only, because the R1-HK virus did not agglutinate the horse erythrocytes. In short, the sera were pretreated with receptor-destroying enzyme, heat inactivated (56 °C, 30') and adsorbed onto either chicken or horse erythrocytes. Sera were incubated with 4 hemagglutinating units of virus for 1 hour and then left to hemagglutinate 0.5% chicken erythrocytes or 1% horse erythrocytes during 1 or 2 hours at room temperature, respectively. In the VN assay, twofold serum dilutions were incubated with 10^2 $TCID_{50}$ of the homologous virus during 1 hour. Next, 100 µl of MDCK cells were added at a concentration of 300,000 cells/ml and after 24 hours the plates were fixed. Lack of infection was confirmed by immunocytochemical staining. For the IPMA assay, monolayers of MDCK cells were inoculated with 10^3 $TCID_{50}$ per well and fixed after 24 hours. They were incubated with twofold dilutions of the swine sera followed by incubation with goat anti-swine peroxidase (De Vleeschauwer et al. 2009). The influenza A virus antibody test kit®, purchased from IDEXX Laboratories and carried out following the manufacturer's instructions, was a blocking ELISA. If NP antibodies are present in the serum sample to be tested, they will prevent the conjugate from binding with the nucleoprotein that is adsorbed to the ELISA plate and color development will be blocked. Starting dilutions of the sera in the serological assays were 1:2 in VN, 1:5 in IPMA and 1:10 in HI and NP-ELISA. All sera were tested in duplicate. Antibody titers were expressed as the reciprocal of the highest serum dilutions that completely inhibited virus replication in MDCK cells (VN assay), that stained influenza infected MDCK cells (IPMA) and that inhibited hemagglutination (HI assay). Results from the NP-ELISA were expressed as S/N ratio. The S/N response is the ratio of the sample optical density (OD) reading to the kit negative control OD reading

4.1.3.7. Lectin histochemistry

The pronounced tropism of R2-HK for the olfactory part of the nasal mucosa triggered us to study the receptor expression in this tissue. Siaα2-6Gal distribution was examined using a digoxigenin labelled Sambucus nigra agglutinin (SNA). Two different isoforms of the Maackia amurensis agglutinin, MAA-I and MAA-II were applied to identify the α2-3 linked Sia moiety of the receptor. Staining was developed with New Fuchsin.

4.1.3.8. Data analyses

Replication differences between viruses in the explants were compared by a paired t-test. P <0.05 was considered significant. The average virus excretion in the nasal swabs from each group of pigs was determined by calculating the area under the curve (AUC). To compare the AUC between groups, the 2 sample Mann-Witney test was used and P <0.05 was considered significant. All analyses were performed with Prism5, version 5.0c.

4.1.4. Results

4.1.4.1. Replication in porcine respiratory explants

Results from replication studies in porcine respiratory explants are shown in Figure 1. Though there was a clear dose-dependent effect, virus replication in NE and TE was comparable, it was poor for dk/It/06, moderate for R2-HK, high for R1-HK and highest for sw/Fl/1/98. In BE, R1-HK and sw/Fl/1/98 showed equally high virus titers (P = 0.92). Replication of R2-HK and dk/It/06 in BE was also increased compared to NE and TE, but remained significantly different from R1-HK (P = 0.008 and P = 0.010) and sw/Fl/98 (P = 0.019 and P = 0.009) respectively when 10^6 PFU was administrated. In LE, in contrast, virus titers did not significantly differ between the 4 viruses at the inoculation doses of 10^6 and 10^5 PFU.

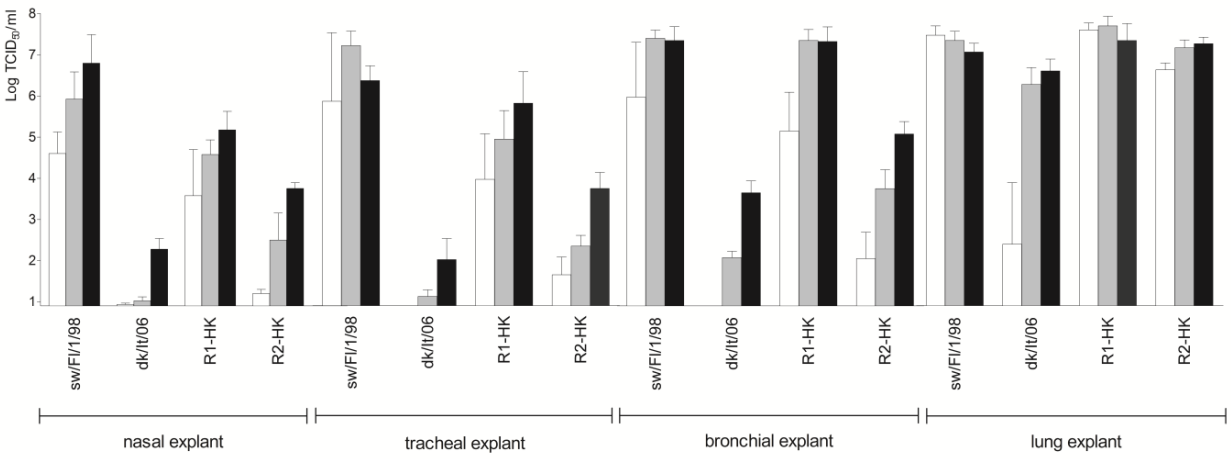

Figure 1: Virus yield in the supernatant of porcine respiratory explants at 48 hpi. Three different inoculation doses were assessed for each virus: 10^4 (white bars), 10^5 (grey bars) and 10^6 PFU (black bars). Bars show the mean and standard deviation of 4 repeats. Swine/Flanders/1/98 (sw/Fl/1/98) and duck/Italy/3139-1/06 (dk/It/06) represent a swine-adapted and non swine-adapted influenza virus respectively.

4.1.4.2. Transmission studies

An overview of the nasal virus shedding in all groups is depicted in Figure 2. After inoculation with R1-HK, all pigs shed virus in nasal swabs until 6 dpi. Virus titers showed little individual variation between pigs, resulting in an average AUC of 23.1. The 6 contact animals in this group also excreted

virus, starting at 1 to 3 dpc. The duration of shedding was similar to that of the inoculated pigs, but the average AUC for the contact group was only 17.4. This was due to very low AUC values in 3 out of 6 pigs.

All R2-HK inoculated pigs were positive for virus excretion. Their excretion profile was similar to that of R1-HK inoculated pigs in terms of start and duration of viral shedding, but the average AUC was only 15.7 (significantly lower than in the R1-HK inoculated group). Four out of 6 pigs showed a remarkable drop in virus excretion at 2 and 3 dpi. By 4 dpi the nasal shedding in all 6 pigs had increased again to levels between 3.8 and 5.3 $\log_{10}$ $TCID_{50}$/ml. In contrast, none of the contact animals in the R2-HK group had detectable virus titers in nasal swabs.

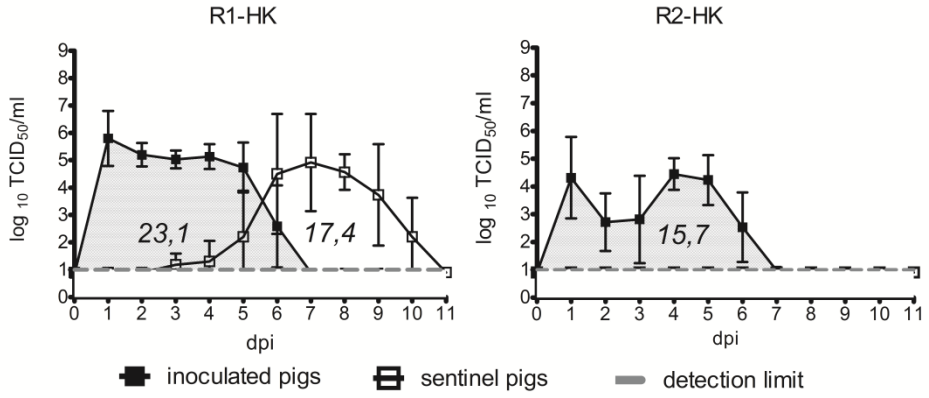

Figure 2: Nasal virus shedding of R1-K (left panel) and R2-HK (right panel) influenza viruses of inoculated and contact pigs. The virus titers are shown as mean ± standard deviation. R2-HK was undetectable in nasal swabs of all contact pigs. The numbers in italics represent the area under the curve for each group of pigs.

4.1.4.3. Pathogenesis study

Out of 11 tissues analysed for virus titers, only 1 was negative during 5 subsequent days for R1-HK compared to 4 negative tissues for R2-HK. The bulbus olfactorius tested negative for both R1-HK and R2-HK, while the tonsil, the right diaphragmatic and the left diaphragmatic lung lobes were also negative for R2-HK. With the exception of the olfactory part of the nasal mucosa, the average virus titers obtained for R2-HK were at least 20-fold lower than for R1-HK. The virus titers obtained for R2-HK were particularly low in bronchus and lungs, with the exception of those in the accessory lung lobe (Figure 3).

4.1.4.4. Serological examination

The results of the serological assays in the R1-HK and R2-HK groups are shown in Table 1 and 2 respectively. In the HI test performed with 0.5% chicken red blood cells (RBC) all R1-HK inoculated

and contact pigs had seroconverted by 14 dpi/dpc (titers 10-40). None of the 12 pigs of the R2-HK group reacted in this assay. In contrast, in the HI test with 1% horse RBC, all R2-HK inoculated pigs had antibody titers (10-40) by 14 dpi but none of the contact animals showed seroconversion. The VN test largely confirmed the results of the HI test but antibody titers for the R2-HK group were significantly lower than for the R1-HK group, even by 28 dpi. One of the 6 contact pigs in the R2-HK group showed seroconversion in both the NP-ELISA and IPMA assay. The IPMA gave the highest number of seropositive animals in both R1-HK and R2-HK groups by 14 dpi.

Table 1: Antibody responses in pigs directly inoculated with R1-HK virus and in contact pigs. The number of positive pigs among the total of 6 pigs is shown. Range of antibody titers in positive pigs are expressed as the reciprocal of the serum dilutions for VN, IPMA and HI assays and as S/N ratio for the NP-ELISA. S/N ratios ≥ 0,6 are considered negative.

	Number of serologically positive pigs and range of antibody titers (in parentheses)					
	Directly inoculated pigs Days post inoculation			Contact pigs Days post contact		
Assay	0	14	28	0	14	28
HI (chicken RBC)	0 (<10)	6 (10-40)	6 (10-40)	0 (<10)	6 (10-40)	6 (10-40)
VN	0 (<2)	6 (24-64)	6 (12-64)	0 (<2)	6 (16-96)	6 (32-64)
NP-Elisa	0 (≥ 0.6)	2 (0.565-0.508)	6 (0.584-0.445)	0 (≥ 0.6)	4 (0.529-0.427)	5 (0.524-0.377)
IPMA	0 (<5)	6 (1280-5120)	6 (1280-10240)	0 (<5)	6 (640-5120)	6 (1280-5120)

Table 2: Antibody responses in pigs directly inoculated with R2-HK virus and in contact pigs. The number of positive pigs among the total of 6 pigs is shown. Range of antibody titers in positive pigs are expressed as the reciprocal of the serum dilutions for VN, IPMA and HI assays and as S/N ratio for the NP-ELISA. S/N ratios ≥ 0,6 are considered negative.

	Number of serologically positive pigs and range of antibody titers (in parentheses)					
	Directly inoculated pigs Days post inoculation			Contact pigs Days post contact		
Assay	0	14	28	0	14	17
HI (chicken RBC)	0 (<10)	0 (<10)	0 (<10)	0 (<10)	0 (<10)	0 (<10)
HI (horse RBC)	0 (<10)	6 (10-20)	5 (20-40)	0 (<10)	0 (<10)	0 (<10)
VN	0 (<2)	4 (2-6)	6 (3-12)	0 (<2)	0 (<2)	0 (<2)
NP-Elisa	0 (≥ 0.6)	6 (0.581-0.250)	6 (0.538-0.343)	0 (≥ 0.6)	1 (0.458)	1 (0.565)
IPMA	0 (<5)	6 (640-2560)	6 (1280-5120)	0 (<5)	1 (320)	1 (640)

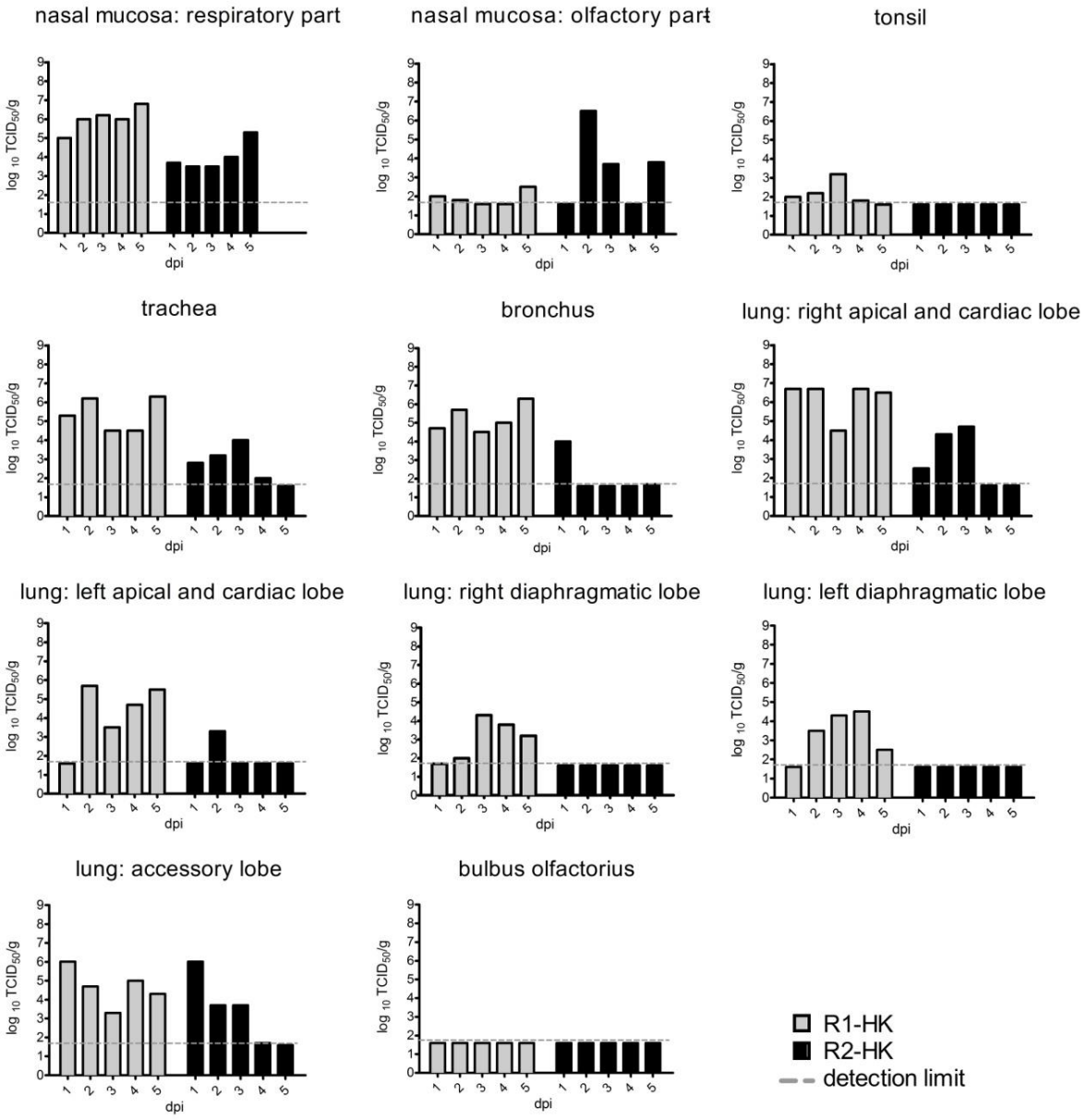

Figure 3: Replication of R1-HK (grey bars) and R2-HK (black bars) influenza virus in various parts of the respiratory tract and in the bulbus olfactorius of pigs during 5 subsequent days post inoculation. Each bar represents the virus titer of an individual pig. The thin dashed line represents the detection limit

4.1.4.5. Lectin histochemistry

There was strong binding of both MAA-I and MAA-II to the epithelial lining of the olfactory part of the nasal mucosa (indicated by the arrows in Figure 4A and B), indicating the presence of both N- and O-linked Siaα2-3 linked glycans. Siaα2-6Gal was not detected in any of the epithelial cell layers as there was no binding by SNA (Figure 4C).

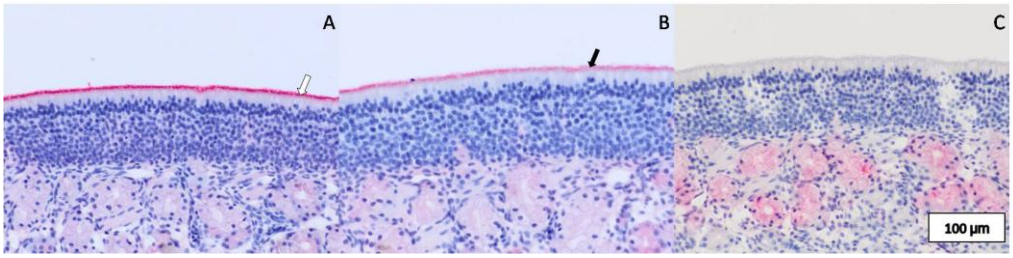

Figure 4: Tissue binding of *Maackia amurensis* agglutinin I (A), *Maackia amurensis* agglutinin II (B) and *Sambucus nigra* agglutinin (C) in the porcine olfactory part of the nasal mucosa. Siaα2-3Galβ1-4Glc (A) was abundantly expressed on the epithelial lining, Siaα2-3Galβ1-3Gal (B) moderately (indicated by the arrows). Sia α2-6Gal (C) was not detected in any of the epithelial cell layers.

4.1.5. Discussion

Techniques such as reverse genetics, enabling the generation of large numbers of mutant or reassortant influenza viruses, have emphasized the need for reliable *ex vivo* tools to study these viruses and to predict their behaviour *in vivo*. Since pigs play an important role in influenza ecology and zoonotic transmission they are the species of choice to investigate the molecular determinants, both at viral and host levels, of inter- and intraspecies transmission. We have previously shown that replication efficiency of influenza viruses isolated from porcine, human and avian hosts differs significantly in NE, TE and BE but not in LE (Van Poucke et al. 2010). The restricted replication of AIVs in tissues of the upper respiratory tract was in line with the limited expression of Siaα2-3Gal in these tissues (Nelli et al. 2010, Trebbien et al. 2011). Using R1-HK and R2-HK viruses that differ solely by two amino acids in the receptor binding site, we have examined the usefulness of this *ex vivo* model of the porcine respiratory tract to assess replication differences between viruses with genetic modifications. The avian-like R2-HK replicated less efficiently than the human-like R1-HK in NE, TE and BE. On the other hand, virus titers of R2-HK were consistently higher than those of dk/It/06. This finding suggests that the two viruses could still differ in receptor binding properties or that the low replication level of the entirely AIV was not only due to a non-optimal receptor binding specificity. A similar observation was the lower replication of R1-HK compared to sw/Fl/1/98 in NE and TE, despite the human-like receptor specificity of both viruses. A reduction of the inoculation dose from 10^6 to 10^4 PFU resulted in a stronger drop of virus replication of R2-HK than of R1-HK, suggesting a lower minimal infectious dose of the latter virus. Roberts and coworkers indeed showed that a similar A/Victoria/3/75 mutant virus with avian-like receptor specificity required a 40-fold higher infectious dose in ferrets than the wild type virus (Roberts et al. 2011). To assess whether the replication differences observed in these explants were biologically relevant *in vivo*, we used the recombinant viruses for transmission and pathogenesis studies in pigs.

Although seroconversion was observed in 1 out of 6 contact pigs of the R2-HK group, our results indicate that a switch in the receptor preference of H3 from Siaα2-6Gal to Siaα2-3Gal was sufficient to block efficient transmission in pigs, as this virus was not isolated in any of the nasal swabs from contact animals. In previous studies in ferrets and guinea pigs intraspecies transmission was also shown to be influenced by receptor binding preferences of viruses (Tumpey et al. 2007, Gao et al. 2009, Pappas et al. 2010). Tumpey et al. (2007) showed that a switch towards an avian-virus-like receptor preference of the 1918 H1N1 pandemic virus abolished airborne transmission between ferrets. Pappas et al. (2010) have also shown that an early isolate of the 1957 H2N2 pandemic with avian-virus-like receptor specificity did not efficiently spread between ferrets. A Q226L mutant with human-virus-like receptor specificity, which emerged during the course of their experiment, was transmissible even through respiratory droplets (Pappas et al. 2010). When compared to R1-HK, the nasal virus shedding of R2-HK inoculated pigs was characterized by overall lower virus titers, a temporary drop at 2 and 3 dpi and an identical duration. The lower AUC of R2-HK, representing the overall viral load built up in the stable, could at least in part explain for the lack of transmission. To examine this possibility we increased the inoculation dose of R2-HK to 10^8 PFU in a preliminary experiment. As the AUC of R2-HK was not significantly increased (AUC=18) under these conditions, the hypothesis could not be confirmed. In literature, data on the role of the virus excretion level on transmission efficiency are contradicting. Mubareka et al. (2009) showed that reduced virus shedding of a human H1N1 compared to a human H3N2 influenza virus was associated with less efficient transmission in guinea pigs. In another transmission study in ferrets a human H3N2 and an avian H5N1 virus achieved similar peak mean titers in nasal washes but only the human virus transmitted efficiently (Maines et al. 2006).

The pathogenesis study confirmed the hampered replication potential of R2-HK in porcine tissues, except for one particular region, the olfactory part of the nasal mucosa. The lower replication of R2-HK could be the result of an imbalance between the receptor preference of HA and substrate specificity of NA. However, Scull et al. (2009) showed that A/Victoria/3/75 with L226Q and S228G mutations in the HA was not significantly less fit than its wild type counterpart as both viruses infected similar numbers of human ciliated airway epithelium cells and replicated to comparable peak titers when incubated at 37 °C. In the LE we observed similar peak virus titers for R2-HK and R1-HK, indicating that the virus itself is replication-competent. Another explanation for the limited R2-HK replication could be a restricted binding of this virus to Sia receptors on target cells. This hypothesis is in agreement with our observations in the explants. R2-HK preferably binds Siaα2-3Gal and the expression of this linkage is absent or limited in NE, TE and BE (Van Poucke et al. 2010). Still, the expected enhanced tropism of R2-HK for the lungs, based on replication in LE and the Siaα2-3Gal

in this organ, was not observed *in vivo*. This may result from a difference in exposure of the tissues to the inoculum. In the LE virus is directly brought onto the tissue, while it must overcome several barriers to reach the LRT after intranasal inoculation. Furthermore we demonstrated a difference in predilection for the olfactory part of the nasal mucosa between R2-HK and R1-HK. The preference of AIVs for this particular tissue was noticed before in pathogenesis studies in our lab (De Vleeschauwer et al. 2009, unpublished data). Here we have shown a correlation with the absence of Siaα2-6Gal and the abundant expression of Siaα2-3Gal on the epithelial lining. Despite a lack of Siaα2-6Gal, swine/Belgium/1/98 (H1N1) could successfully infect this olfactory part of the nasal mucosa (De Vleeschauwer et al. 2009), indicating that the SNA lectin may not stain all Siaα2-6 variants or that another receptor mediates the endocytotic uptake of the virus.

In conclusion, our data suggest that Siaα2-6Gal binding is a prerequisite for efficient transmission of influenza viruses in pigs. This observation strengthens the resemblance between pigs and humans in terms of host range restrictions for influenza virus infections. The transmission efficiency was related to the overall replication level *in vivo*, for which the porcine explants can have a predictable value.

4.1.6. Acknowledgments

This work was financed by 7th Framework Programme FLUPIG (FP7-GA 258084). Special thanks go to Lieve Sys, Nele Dennequin, Melanie Bauwens, Zeger Van den Abeele, Bart Ellebaut and Kevin Fung for excellent technical support

4.1.7. References

Choi Y.K., Nguyen T.D., Ozaki H. et al. (2005). Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol 79: 10821-10825.

Claas E.C.J., Kawaoka Y., De Jong J.C. et al. (1994). Infection of children with avian-human reassortant influenza virus from pigs in Europe. Virology 204: 453-457.

Connor R.J., Kawaoka Y., Webster R.G. et al. (1994). Receptor specificity in human, avian and equine H2 and H3 influenza virus isolates: Virology 205: 17-23.

De Vleeschauwer A., Atanasova K., Van Borm S. et al. (2009a). Comparative pathogenesis of an avian H5N2 and a swine H1N1 influenza virus in pigs. PLoS ONE 4: doi:10.1371/journal.pone.0006662.

De Vleeschauwer A., Van Poucke S., Braeckmans D. et al. (2009b). Efficient transmission of swine-adapted but not wholly avian influenza viruses among pigs and from pigs to ferrets. JID 200: 1884-1892.

De Vleeschauwer A.R., Van Poucke S.G., Karasin A..I. et al. (2010). Cross-protection between antigenically distinct H1N1 swine influenza viruses from Europe and North America. Influenza Other Respi Viruses 5: 115-122

Gao Y., Zhang Y., Shinya K. et al. (2009). Identification of Amino Acids in HA and PB2 Critical for the Transmission of H5N1 Avian Influenza Viruses in a Mammalian Host. PLoS Pathogens 5: doi: 10.1371/journal.ppat.1000709.

Garten R.J., Davis C.T., Russel C.A. et al. (2009). Antigenic and genetic characteristics of swine-origin 2009 A (H1N1) influenza viruses circulating in humans. Science 325: 197-201.

Hoffmann E., Neumann G., Kawaoka Y. et al. (2000). A DNA transfection system for generation of influenza A virus from eight plasmids. PNAS 97: 6108-6113.

Ito T., Couceiro J.N., Kelm S. et al. (1998). Molecular basis for the generation in pigs of influenza viruses with pandemic potential. J Virol 72: 7367-7373.

Kida H., Ito T., Yasuda J. et al. (1994). Potential for transmission of avian influenza viruses to pigs. J Gen Virol 75: 2183-2188.

Kilbourne E.D. (2006). Influenza pandemics of the 20th century. Em Infect Dis 12: 9-14.

Lipatov A.S., Kwon Y.K., Sarmento L.V. et al. (2008). Domestic pigs have low susceptibility to H5N1 highly pathogenic avian influenza viruses. PLoS Pathogens 4: doi:10.1371/journal.ppat.1000102.

Ma W., Lager K.M., Vincent A.L. et al. (2009). The role of swine in the generation of novel influenza viruses. Zoonoses and public health 56: 326-337.

Maines T.R., Chen L., Matsuoka Y. et al. (2006). Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. PNAS 103: 12121-12126.

Matrosovich M., Tuzikov A., Bovin N. et al. (2000). Early alterations of the receptor-binding properties of H1, H2 and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol 74: 8502-8512.

Matrosovich M.N., Matrosovich T.Y., Gray T. et al. (2004). Human and avian influenza viruses target different cell types in cultures of human airway epithelium. PNAS 101: 4620-4624

Matrosovich M., Matrosovich T., Uhlendorff J. et al. (2007). Avian-virus-like receptor specificity of the hemagglutinin impedes influenza virus replication in cultures of human airway epithelium. Virology 361: 384-390.

Mubareka S., Lowen A.C., Steel J. et al. (2009). Transmission of influenza virus via aerosols and fomites in the guinea pig model. JID 199: 858-865.

Nelli R.K., Kuchipudi S.V., White G.A. et al. (2010). Comparative distribution of human and avian type sialic acid influenza receptors in the pig. Vet Res 6: doi:10.1186/1746-6148-6-4.

Pappas C., Viswanathan K., Chandrasekaran A. et al. (2010). Receptor Specificity and Transmission of H2N2 Subtype Viruses Isolated from the Pandemic of 1957. PLoS ONE 5: doi:10.1371/journal.pone.0011158.

Reed L.J. and Münch H. (1938). A simple method of estimating fifty per cent endpoints. Am J Hygiene 27: 493-497.

Roberts K.L., Shelton H., Scull M. et al. (2011). Lack of transmission of a human influenza virus with avian receptor specificity between ferrets is not due to decreased virus shedding, but rather a lower infectivity in vivo. J Gen Virol 92: 1822-1831.

Scholtissek C., Bürger H., Kistner O. et al. (1985). The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology 147: 287-294.

Scull M.A., Gillim-Ross L., Santos C. et al. (2009). Avian influenza virus glycoproteins restrict virus replication and spread through human airway epithelium at temperatures of the proximal airways. PLoS Pathogens 5: doi:10.1371/journal.ppat.1000424.

Shinya K., Ebina M., Yamada S.et al. (2006). Influenza virus receptors in the human airway. Nature 440: 435-436.

Shortridge K.F., Zhou N.N., Guan Y. et al. (1998). Characterisation of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 252: 331-342.

Trebbien R., Larsen L.E., Viuff B.M. et al. (2011). Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virol J doi:10.1186/1743-422X-8-434.

Tumpey T.M., Maines T.R, Van Hoeven N. et al. (2007). A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science 315: 655-659.

Van Poucke S.G., Nicholls J.M., Nauwynck H.J. et al. (2010). Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. Virol J 7: doi:10.1186/1743-422X-7-38.

Vines A., Wells K., Matrosovich M. et al. (1998). The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. J Virol 72: 7626-7631.

Yao L., Korteweg C., Hsueh W. et al. (2008). Avian influenza receptor expression in H5N1-infected and noninfected human tissues. FASEB J 22: 733-740.

4.2.

Does a switch in receptor binding specificity of A/Hong Kong/1/68 (H3N2) affect porcine respiratory tract cell tropism and interactions with mucus or surfactant proteins?

Van Poucke S.M., Nicholls J., Van Breedam W., van Eijk M., Billiau V., Matrosovich M., Haagsman H.P., Van Reeth K.

Manuscript in preparation

4.2.1. Abstract

Several researchers have provided evidence that avian(-like) influenza viruses have a low replication potential in the porcine respiratory tract. These observations have been attributed to a low Siaα2-3 receptor expression in the pig and the preferable infection of other cell types by avian influenza virus compared to host adapted viruses.

In this study we characterized the R1-HK and R2-HK viruses from chapter 4.1. in terms of glycan binding profile, expression of surface glycoproteins and their release capacities from MDCK cells. Furthermore, the types of cells predominantly infected by both viruses in the pig nasal mucosa, the trachea, bronchi and lungs were quantified. In a last part, the interaction of the viruses with non-specific antiviral components such as mucus and porcine surfactant proteins SP-D and SP-A were studied.

The virus characterization confirmed that the 2 amino acid mutations in the R2-HK hemagglutinin caused a switch in receptor binding preference which was not accompanied by a compromised surface glycoprotein expression nor a reduced release of virus particles. While the cell tropism study displayed a difference in the number of infected cells no distinction could be made in the type of cells infected. Significant differences, however, in the way both viruses were interacting with mucus and SP-D were observed. We hypothesize that although mucus and SP-D have an anti-viral effect, the Siaα2-6 binding viruses might have an advantage by binding stronger to these components than Siaα2-3 binding viruses.

4.2.2. Introduction

In chapter 4.1, using recombinant viruses R1-HK and R2-HK, we demonstrated that the acquisition of a human-like receptor preference is a prerequisite for efficient replication and transmission of influenza viruses in the pig. R1-HK harbored all genes from the original pandemic virus A/Hong Kong/1/68 (H3N2) while R2-HK only differed by L226Q and S228G mutations in the HA and a conversion to an avian-like receptor specificity. Based on earlier findings, one might postulate that the low replication capacity of R2-HK was due to a poor fit with the cellular Sia receptors present on the epithelia of the porcine respiratory tract. In addition, several researchers have provided indications that human (-like) and avian (-like) viruses target different cell types. Yet, depending on the system used, rather contradicting results have been obtained. The earliest research by Baum and Paulson (1990) and Couceiro et al. (1993) showed that attachment of human influenza viruses was confined to ciliated cells in human tracheal tissue sections. In primary human tracheobronchial epithelial cells, human (-like) viruses were repeatedly shown to preferentially infect non-ciliated cells whereas avian (-like) viruses infected ciliated cells (Matrosovich et al 2004a; 2007, Thompson et al. 2006, Wan and Perez 2007). Using primary human tracheal epithelial cells and fresh human tracheal tissues as a control, Ibricevic et al. (2006) confirmed that Siaα2-3Gal was exclusively present on ciliated cells while Siaα2-6Gal was present on both ciliated and goblet cells.

Other findings, like those by Punyadarsaniya et al. (2011) suggested a less strict difference in the cell tropism of viruses with a distinct receptor binding preference. These researchers have used pig lung cultures to assess the replication of one swine influenza virus (H3N2) and two avian isolates (H7N7 and H9N2). They particularly focused on the cell tropism of these influenza viruses in the bronchiolar epithelium. Although lectin histochemistry binding revealed an identical distribution of Sia variants on the epithelial cells as that observed by Ibricevic et al. (2006) in human cells, the cell tropism results were different. These authors performed double stainings with HB-65 (binding to the influenza NP) and anti-β-tubulin (marker for ciliated cells) or anti-mucin5Ac (marker for goblet cells and mucus). They concluded that the bronchial ciliated cells were infected by all viruses and goblet cells predominantly by the swine influenza isolate and the avian H7N7. The latter is rather unexpected as no Siaα2-3Gal was detected on the goblet cells. So far, no studies were undertaken to assess the correlation between the receptor binding preference of an influenza A virus and the cell tropism *in vivo.*

However, physical barriers present in the respiratory tract, including mucus and surfactant proteins-D (SP-D) and SP-A, could also play a role in the outcome of virus infection. Before any influenza virus

can reach a susceptible epithelial cell with the proper receptor it will have to overcome these non-specific barriers. The relative contribution of mucus as a defense mechanism for influenza virus infection has not been studied in great detail. Mucins decorated with Sia, produced in goblet cells and submucosal glands, are supposed to act as decoy receptors by competitively inhibiting HA-mediated cell adsorption and infection (Matrosovich and Klenk 2003). Formal proof of concept was provided recently (Ehre et al. 2012) by using a transgenic mouse model that over-expressed Siaα2-3Gal rich Mucin5Ac. Transgenic animals inoculated with PR8 H1N1, which has a Siaα2-3 binding preference, had significantly lower virus titers in the lungs compared to normal mice. The authors hypothesized that this virus was more efficiently expelled from the respiratory tract by means of the mucociliary route. An additional *in vitro* test, measuring the remaining infectivity of PR8 after incubation with bronchio-alveolar lavage fluid (BALF) from transgenic mice, showed a significant reduction compared to incubation with PBS or BALF from normal mice. Since neuraminidase treatment of the transgenic BALF restored infectivity, the authors ascribed the antiviral effect (at least partially) to binding with Sia. Roberts et al. (2011) applied a similar assay to screen the antiviral activity of nasal ferret washes against two viruses that differed only by L226Q and S228G mutations in the HA and that had their receptor specificity converted to avian-like. They incubated the viruses with the nasal fluids and assessed the percentage of remaining infectious particles by plaque titration of these mixtures. Lectin histochemistry on the nasal washes additionally showed the abundant presence of Siaα2-6Gal linkages. In their test, the wild type virus with human receptor binding phenotype was more efficiently blocked compared to the avian-like variant. These results seemed contradictory because the wild type virus replicated more efficient in ferrets than the avian-like variant. The authors suggested that a stronger binding of an influenza virus to mucus components might be beneficial by helping the virus to penetrate the physical barrier.

The interaction mechanisms between influenza viruses and SP-D or SP-A, on the other hand, have been better studied. Porcine SP-D contains a carbohydrate recognition domain (CRD) that interacts directly with N-linked high mannose carbohydrates on HA and NA in a Ca^{2+} dependent manner. A unique feature of porcine SP-D is the additional presence of terminal α2-6 linked Sia on the CRD, which allows interaction with HA. SP-A exclusively possesses sialylated α2-6 and α2-3 N-linked oligosaccharides that interfere with HA-binding to the cell receptor (van Eijk et al. 2002, Haagsman et al.2003, van Eijk et al. 2012). The antiviral activity, in particular for SP-D has been proven in several infectivity reduction tests (van Eijk et al. 2004, Hillaire 2011 et al.). Also in these tests, the viruses with a Siaα2-6Gal binding preference were more strongly inhibited.

This study aimed to define the cell tropism of viruses with distinct receptor binding preferences in the porcine respiratory tract. By performing infectivity reduction tests with mucus and surfactant

proteins we obtained some insights on how the virus binding preference influences the interaction with non-specific host defense mechanisms.

4.2.3. Methods

4.2.3.1. Characterization of recombinant viruses

Western blot

To assure that the mutant R2-HK virus expressed HA glycoproteins on its virion as efficiently as R1-HK, we compared for both viruses the quantity of HA relative to the amounts of other viral proteins, such as NP. Density gradient ultracentrifugation purified virus stocks were diluted to equal protein concentrations of 0.5 mg/ml proteins (as determined by nano-drop technology) and analyzed by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (Nobusawa et al. 2000). Proteins reduced by addition of 5% β mercapto-ethanol, were separated using a 8% separating gel with a 4% stacking gel. Gels were run at 200 V for approximately 40 minutes. After protein separation by electrophoresis, samples were transferred to a Hybond-P (GE Healthcare) membrane, a hydrophobic polyvinylidene difluoride membrane. After blocking non-specific binding sites on the membrane with dried skimmed milk in PBS, the membrane was incubated with a mixture of polyclonal antibodies derived from hyperimmune serum. This hyperimmune serum was obtained by intranasal inoculation of 2 pigs with R1-HK or R2-HK respectively. Four weeks later, the pigs were boosted by subcutaneous and intramuscular administration of a suspension containing the respective virus stock and Freunds adjuvant complete (Sigma Life Sciences) in a 1:1 ratio. After collection of the sera from both pigs, the polyclonal antibodies were purified with a sepharose protein G column (GE healthcare) and mixed. Horseradish peroxidase-labeled goat anti-swine was used for chemiluminescence detection in the presence of enhanced chemiluminescence (ECL) (GE healthcare).

Attachment-release assay

As we observed that the average plaque size produced in MDCK cells by R1-HK was larger than that for R2-HK (results not shown), we assessed whether the amino acid substitutions L226Q and S228G in the HA RBS caused a functional disbalance with the NA-activity. We investigated the release of R1-HK and R2-HK from fixed MDCK cells in 24-well plates at different time points. Since the NA activity is blocked at 4 °C we allowed 13000 PFU of R1-HK and R2-HK virus, diluted in 300 µl of medium, to attach to fixed MDCK-cells for 1 hour at 4 °C. Subsequently, still at 4 °C, the inoculum was removed and 1 ml of new medium was added to each well. To allow release of attached viruses by NA, the cells were transferred to 37 °C and the supernatant was collected after 3, 7, 10, 20 and 30 minutes of incubation. The number of infectious viruses in this supernatant was determined by plaque

titration. In order to judge how much virus from the initial inoculum were attaching to the MDCK cells, the inoculum removed after one hour of incubation at 4 °C was also plaque titrated. To show that the presence of virus in the supernatant was specifically due to NA activity, 3 wells per virus were also incubated during 30 minutes at 4 °C.

Glycan microarray screening

The viruses were submitted to the CFG (Consortium for Functional Glycomics) for binding to a library of 610 different glycans. These glycans were printed onto N-hydroxy-succinimide-activated glass microscope slides. Attachment of the Alexa 488 labelled virus samples to diverse glycan structures was detected with a PerkinElmer Scan Array scanner. The quantity of virus, tested in this assay, was expressed in hemagglutinating units per ml (determined with turkey red blood cells).

4.2.3.2. Tissues derived from infected pigs

Six- to 8-week-old pigs were obtained from a commercial herd serologically negative for influenza as examined by hemagglutination inhibition (HI) assay, by a competitive anti-influenza A nucleoprotein enzyme immunoassay (Idexx Laboratories) and by immunoperoxidase monolayer assay (IPMA). Swine/Belgium/1/98 (H1N1), swine/Flanders/1/98 (H3N2) and swine/Gent/7625/99 (H1N2) were used in the HI and IPMA assay. Two groups of pigs were housed in separate high-efficiency particulate air-filtered isolation units. At arrival they were treated intramuscularly with ceftiofur (Naxcel®, Pfizer-1ml/20 kg body weight). All experiments were authorized by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine, Ghent University.

Two days after intranasal inoculation with 10^6 PFU of R1-HK or R2-HK animals were euthanized and the following formalin fixed and paraffin embedded samples were collected: nasal mucosa respiratory part, nasal mucosa olfactory part, tonsils, trachea, bronchus and cardiac lung lobes.

4.2.3.3. NP immunohistochemistry

This staining was performed on *in vivo* tissues only. IHC methods were similar as described in 3.3.6., apart from some slight modifications. Ten sections of 4 µm thick cut from different areas in the sample were analyzed. Enzyme-induced antigen retrieval (AR) was accomplished by incubating the deparaffinized and rehydrated sections with 0.1% pronase (Roche) at 37 °C for four minutes. Endogenous peroxidase and biotin activity were blocked by incubation with 3% H_2O_2 (15 min) and the biotin/avidin blocking kit (10 min, Vector) at room temperature respectively. Incubation with the anti-influenza A nucleoprotein (NP) monoclonal antibody at a 1:25 dilution was performed at room temperature for 90 minutes. Slides were incubated with biotinylated goat anti-mouse IgG (Jackson,

1:500) for 30 minutes at room temperature. After another wash, they were incubated with the avidin-biotin complex for 30 minutes (1:50), developed with AEC and counterstained with hematoxylin. Slides were analyzed with the automated Olympus BX61 light microscope.

4.2.3.4. Double staining with cell markers

To confirm the results obtained by IHC on *in vivo* tissues, we performed double stainings on NE. The explants, inoculated with 10^6 PFU R1-HK were formalin fixed at 48 hpi and paraffin embedded. While HB-65 (ATT, 1:25) was directed against the NP, ciliated cells and goblet cells were identified by using beta-tubulin (Abcam, 1:200) and Mucin5Ac (Invitrogen, 1:100) antibodies respectively. Methods used for dewaxing, rehydratation, pronase pretreatment and HB65 incubation steps were identical for both cell markers. Because the beta-tubulin incubation step was preceded by microwaving the sections at 95 °C during 7 mintues in 10mM citrate buffer pH 6.0, we first added biotinylated goat anti-mouse IgG (Jackson lab, 1:500) to bind and to protect the temperature sensitive HB-65 antibody. Development finally took place by incubation during one hour at room temperature with a mixture of Texas Red avidin-D (Vectorlabs, 1:200) and fluorescein goat anti-rabbit IgG (Invitrogen, 1:200). These were directed against the biotinylated goat anti-mouse IgG and the anti-beta-tubulin antibodies respectively. For the mucin5Ac staining color was directly developed with a mixture of goat anti-mouse IgG2a AF594 (1:100) and fluorescein goat anti-mouse IgG1 (Abcam, 1:100) which bound to the HB-65 IgG2a and the anti-mucin IgG1. In all sections nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

4.2.3.5. Mucus infectivity reduction assay

Virus was pre-incubated with airway secretions collected in two diverse ways from slaughterhouse lungs of pigs. The trachea and the first bifurcations were dissected out from surrounding alveolar tissues and flushed with PBS. The expelled mucus (referred to as gel layer) was collected and preserved at 4 °C until use. Next, the trachea was opened and transferred to 37 °C in a humidified box for 3 hours. After this incubation period, the superficial mucus layer was scraped off from the epithelium (referred to as sol layer) and equally preserved at 4 °C. R1-HK and R2-HK viruses were diluted to 130 PFU in 300 µl MDCK medium and pre-incubated for 1 hour at 4 °C with 300 µl of gel layer, sol layer or MDCK medium as a negative control. In triplicate, MDCK cells in six-well plates were infected with either mixture for 1 hour at 37 °C and 5% CO2. The inoculum was removed and monolayers were washed with PBS and overlaid with carboxymethyl cellulose (CMC) as described for plaque assays (Matrosovich et al. 2006). Twenty four hours after inoculation, the cells were fixed and

stained for the presence of the NP. The percentage infectivity remaining with the virus-mucus mixtures compared to incubation with medium alone was calculated.

4.2.3.6. Surfactant protein infectivity reduction assay

To determine the neutralizing capacity of recombinant porcine SP-D (rpSP-D) and pSP-A for R1-HK and R2-HK, an infection reduction assay was set up based on the study by Hillaire et al. (2011). These tests were performed in 3-fold and repeated on 2 different days.

In sterile 96-well U-bottom plates two-fold diltutions of rpSP-D or pSP-A were made in 30 µl DPBS-CM starting from a concentration of 20 µg/ml down to 0.31 µg/ml. The last well only contained 30 µl of DPBS-CM as a negative control. To every well, 30 µl of a standard preparation of 300 PFU R1-HK or R2-HK virus was added. After briefly shaking the plates, they were incubated for 1 hour at room temperature. Subsequently 50 µl of each well was transferred to MDCK-cells washed with DPBS-CM and incubated for 1 hour at 37 °C. The inoculum was removed, the cells washed twice with DPBS containing 5 mM of EDTA and refed with fresh MDCK-medium. Five hours post inoculation, cells were fixed and stained according to methods described in 3.3.4.

4.2.4. Results

4.2.4.1. Virus characterization

On the Western blot (WB) the following proteins from both virus stocks were visualized: HA0, HA1, NP, M1, PA and PB1/2 (Figure 1). The virus stocks of the viruses were diluted to equal protein concentrations of 0.5 mg/ml and a volume of 10 µl was transferred into each slot. The bands obtained for R2-HK were more or less equal to the bands for R1-HK. The ratios of the quantity of NP and HA0 proteins (as analyzed by TotalLab Quant) were undistinguishable (1.2 each). These results indicated that if one of the viruses would express altered levels of HA on the surface, differences could only be minor.

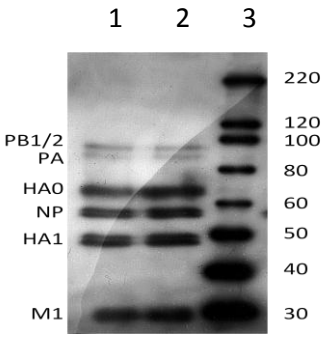

Figure 1: Purified virus particles of R1-HK and R2-HK virus stocks analyzed by western blot. Viral proteins were identified by ECL with anti-R1/R2-HK antibodies and anti-pig horse-radish peroxidase conjugate. Lane 1: R1-HK,

lane 2: R2-HK and lane 3: Magic Mark XP Western Protein Standard (Invitrogen). Values next to the marker are in kDa.

The results from the attach-release assay, as depicted in Figure 2, revealed that the average fraction of the virus inoculum attaching to these cells was lower for R2-HK (4444 PFU) than for R1-HK (5742 PFU). The average number of released virus particles in function of time, on the other hand was higher for R2-HK compared to R1-HK. After 30 minutes incubation at 37 °C, the majority of the attached R2-HK viruses were released (82.5%), while this was only around 35% for R1-HK. When the cells were incubated at 4 °C for 30 minutes a significantly lower number of viruses was released, indicating that most of the observed virus detachment was due to neuraminidase activity.

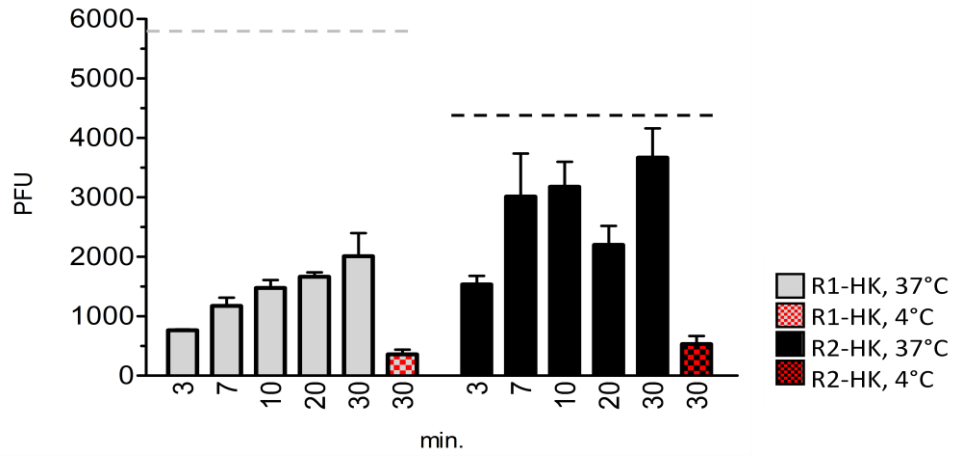

Figure 2: Number of released infectious virus particles (expressed in plaque forming units (PFU)) of R1-HK and R2-HK after different incubation times at 37 °C. Each bar shows the average of 3 repeats with the standard deviation. When the cells were incubated at 4 °C for 30 minutes (min.) a significantly lower number of viruses was released, indicating that most of the observed virus detachment was due to neuraminidase activity. The dotted lines represent the average number of viruses (out of 13000PFU) that are attaching to the MDCK cells.

Binding of R1-HK and R2-HK to a panel of 610 different glycans on a microarray, as shown in Figures 3 and 4 respectively, revealed clear differences between both viruses. The composition of the 35 strongest binding oligosaccharides is displayed in Table 1 and 2 respectively. The vast majority of oligosaccharides that were strongly bound by R1-HK contained terminal α2-6 linked Sia, whereas R2-HK mostly bound to the α2-3 variants. Another difference was identified at the level of the second linkage between Gal and the subsequent saccharide. The R1-HK preferred binding to the β1-4 variants whereas the R2-HK did not seem to differentiate between β1-4 or β1-3 linkages. Comparing Figures 3 and 4 from the glycan arrays showed two peaks of R1-HK binding around the glycan structures with numbers 55-57 and 605-608, corresponding with bi-antennary complex-type N-glycans. Corresponding peaks were missing in the R2-HK graph.

Table 1. Overview of the 35 strongest binding oligosaccharides for R1-HK and their chart numbers.

Chart Number	R1 (5,000HAU/ml)	Average RFU
464	Neu5Aca2-6Galb1-4GlcNAcb1-4Mana1-6(GlcNAcb1-4)(Neu5Aca2-6Galb1-4GlcNAcb1-4(Neu5Aca2-6Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	895
318	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	857
481	Neu5Aca2-6Galb1-4 GlcNAcb1-6(Neu5Aca2-6Galb1-4GlcNAcb1-3)GalNAca-Sp14	839
326	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	807
55	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	724
57	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp24	692
270	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	689
332	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0	667
266	Neu5Aca2-6GalNAcb1-4GlcNAcb-Sp0	651
502	Neu5Aca2-6GalNAcb1-4(6S)GlcNAcb-Sp8	604
267	Neu5Aca2-6Galb1-4(6S)GlcNAcb-Sp8	586
466	Neu5Aca2-6Galb1-4GlcNAcb1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-6Galb1-4GlcNAcb1-4(Neu5Aca2-6Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	572
482	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24	563
606	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	445
269	Neu5Aca2-6Galb1-4GlcNAcb-Sp8	443
347	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6Manb1-4GlcNAcb1-4GlcNAc-Sp12	430
605	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3)GalNAca-Sp14	414
330	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-3GlcNAcb-Sp0	383
271	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0	369
463	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-4)(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	345
268	Neu5Aca2-6Galb1-4GlcNAcb-Sp0	343
56	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Man-a1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	303
348	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3Manb1-4GlcNAcb1-4GlcNAc-Sp12	298
367	Neu5Aca2-6GlcNAcb1-4GlcNAcb1-4GlcNAc-Sp21	280
601	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14	274
320	GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	260
366	Neu5Aca2-6GlcNAcb1-4GlcNAc-Sp21	246
608	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	236
319	Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	223
325	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	165
465	Neu5Aca2-6Galb1-4GlcNAcb1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	153
308	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	101
345	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Mana1-3)Manb1-4GlcNAcb1-4GlcNAc-Sp12	88
602	Neu5Aca2-6Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14	84
462	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-4(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	83

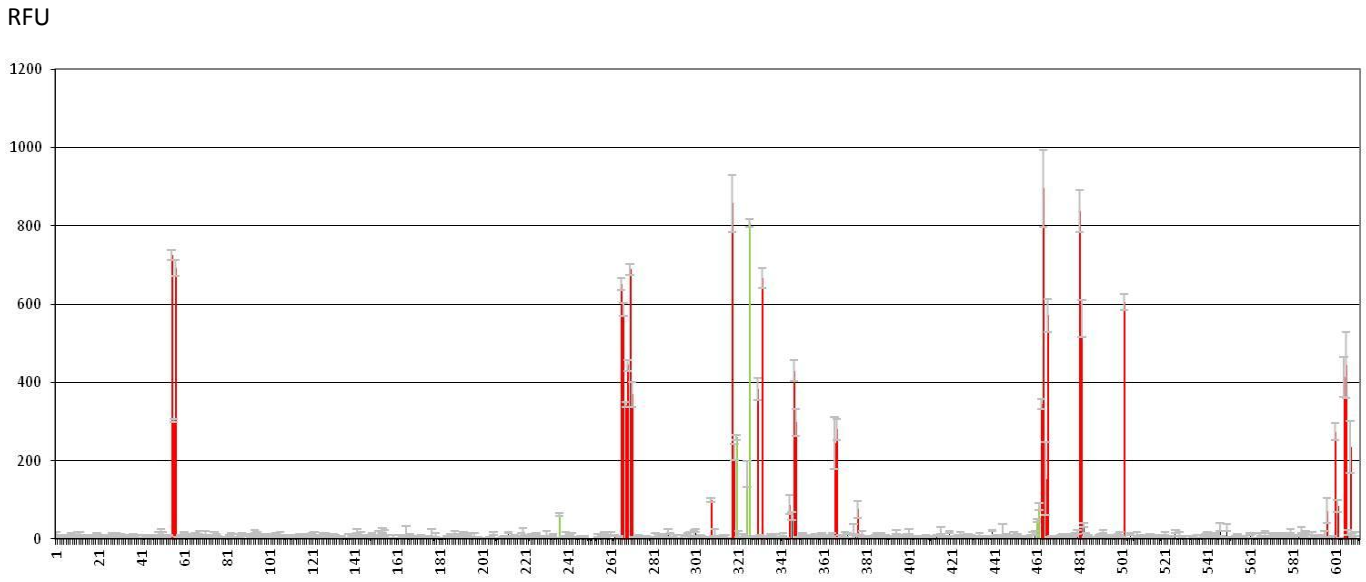

Figure 3: Glycan array analysis of R1-HK as measured by fluorescence intensity (RFU). Error bars represent the mean ± standard deviation of 4 repeats. Structures with terminal Siaα2-6 or Siaα2-3 are in red and green respectively.

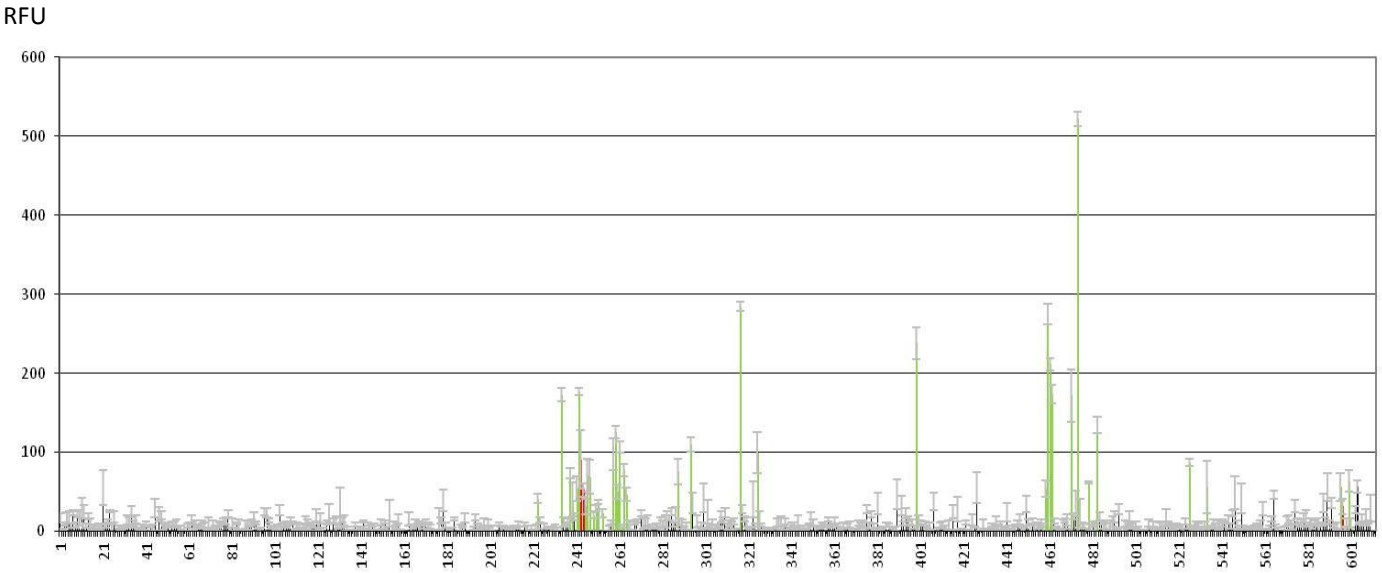

Figure 4: Glycan array analysis of R2-HK as measured by fluorescence intensity (RFU). Error bars represent the mean ± standard deviation of 4 repeats. Structures with terminal Siaα2-6 or Siaα2-3 are in red and green respectively.

Table2. Overview of the 35 strongest binding oligosaccharides for R2-HK and their chart numbers

Chart Number	R2 (1800HAU/ml)	Average RFU
474	Neu5Aca2-3Galb1-3GlcNAcb1-6(Neu5Aca2-3Galb1-3GlcNAcb1-2)Mana1-6(Neu5Aca2-3Galb1-3GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp19	521
317	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-3)GalNAca-Sp14	284
460	Neu5Aca2-3Galb1-4GlcNAcb1-4Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-4(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	275
399	Neu5Aca2-3Galb1-3GlcNAcb1-3GalNAca-Sp14	238
461	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	211
242	Neu5Aca2-3Galb1-3(6S)GalNAca-Sp8	176
462	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-4(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	173
234	Neu5Aca2-3Galb1-3GalNAcb1-4(Neu5Aca2-3)Galb1-4Glcb-Sp0	173
471	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3)GalNAca-Sp14	172
483	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24	135
259	Neu5Aca2-3Galb1-4GlcNAcb-Sp0	125
294	Neu5Aca2-3Galb1-3GlcNAcb1-3Galb1-3GlcNAcb-Sp0	110
261	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0	107
325	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	99
258	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0	97
243	Neu5Aca2-6(Neu5Aca2-3Galb1-3)GalNAca-Sp8	91
526	Neu5Aca2-3Galb1-3GlcNAcb1-2Mana-Sp0	87
263	Neu5Aca2-3Galb1-4Glcb-Sp0	77
288	Neu5Aca2-3Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14	75
238	Neu5Aca2-3Galb1-3(6S)GlcNAc-Sp8	73
246	Neu5Aca2-3Galb1-3GalNAcb1-3Gala1-4Galb1-4Glcb-Sp0	71
247	Neu5Aca2-3Galb1-3GlcNAcb1-3Galb1-4GlcNAcb-Sp0	69
600	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14	64
479	Neu5Aca2-3Galb1-4GlcNAcb1-6GalNAca-Sp14	62
604	GlcNAcb1-6(Neu5Aca2-3Galb1-3)GalNAca-Sp14	56
596	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3)GalNAca-Sp14	56
534	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-2Mana-Sp0	56
590	Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14	55
427	Gala1-3(Fuca1-2)Galb1-4GlcNAcb1-2Mana1-6(Gala1-3(Fuca1-2)Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp22	55
21	GlcNAcb1-6(GlcNAcb1-4)(GlcNAcb1-3)GlcNAc-Sp8	55
459	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	54
241	Neu5Aca2-3Galb1-4(Neu5Aca2-3Galb1-3)GlcNAcb-Sp8	54
244	Neu5Aca2-6(Neu5Aca2-3Galb1-3)GalNAca-Sp14	52
260	Neu5Aca2-3Galb1-4GlcNAcb-Sp8	50

4.2.4.2. Viral antigen distribution *in vivo*

An overview of the number of positive cells counted in 10 sections is given in Table 3, representative pictures of infected tissues are shown in Figure 5. The virus titrations of various organs from pigs inoculated with R1-HK or R2-HK, as described under 4.1.4.3, showed a lower replication level of R2-HK in the majority of the tissues analysed. Here we focused in more detail on the cell types infected *in vivo* by the respective viruses at 2 days after intranasal inoculation.

Firstly we observed that detection of R2-HK infected cells was scarce in all tissues apart from the olfactory part of the nasal mucosa. In the tissues containing a pseudostratified respiratory epithelium, being the nasal mucosa respiratory part and the trachea, ciliated cells were more readily infected than goblet cells by both viruses. When the infection with R1-HK was pronounced, positive basal cells were seen. In the olfactory part of the nasal mucosa, observations were the opposite, displaying more successful infection by R2-HK. The majority of the cells positive for R1-HK and R2-HK NP belonged to the sensory type, although R2-HK also infected sustentacular cells and basal cells rather efficiently. In the tonsils of R1-HK and R2-HK inoculated animals, the lumen of the crypts were filled with plugs of detritus from shed epithelial cells. Especially in the R1-HK group this debris contained numerous NP positive cells. Also within the stratified squamous epithelium, composed of 3 epithelial layers and migrating non-epithelial cells, and within the surrounding diffuse lymphoid tissue, stained cells were detected with eiter virus. In the bronchi no NP positive cells with either virus were detected. Within the lungs, a differentiation was made between infection of bronchi, bronchioles and alveolar tissues. The majority of R1-HK infected cells were located in the bronchioles, although limited amounts of positive cells were also present in alveoli and bronchi. In the latter, no R2-HK infected cells were found. It appeared as most of the R2-HK positive cells were situated in the alveolar tissues. Since only five R2-HK positive cells were counted in the lung this result asks for cautious interpretation.

4.2.4.3. Immunostaining with cell markers

Immunostaining of the NE revealed that the proportion of ciliated cells is notably higher compared to goblet cells along the epithelial lining. In infected goblet cells the NP protein most regurlarly was located at the edge of the mucus plug, while in ciliated cells positivity was detected in the nucleus and/or the cytoplasm (Figure 6).

Table 3 : Immunohistochemical analysis of the cell tropism of R2-HK and R1-HK in the porcine respiratory tract at 2 days post inoculation. The total number of identifiable R1-HK and R2-HK positive cells in 10 sections are shown, to which cell type these positive cells were belonging is displayed as a percentage.

Infected cells	Cells infected with …..virus in the respiratory part of the nasal mucosa	
	R1-HK	R2-HK
# infected cells	115	14
% ciliated cells	80	72
% goblet cells	20	28
% basal cells	0	0

Infected cells	Cells infected with …..virus in the olfactory part of the nasal mucosa	
	R1-HK	R2-HK
# infected cells	3	109
% sustentacular cells	33	18
% sensory cells	67	67
% basal cells	0	15

Infected cells	Cells infected with …..virus in the trachea	
	R1-HK	R2-HK
# infected cells	339	8
% ciliated cells	51	75
% goblet cells	24	25
% basal cells	25	0

Infected cells	Cells infected with …..virus in the lung	
	R1-HK	R2-HK
# infected cells	92	5
% bronchus	16	0
% bronchioles	72	20
% alveolae	12	80

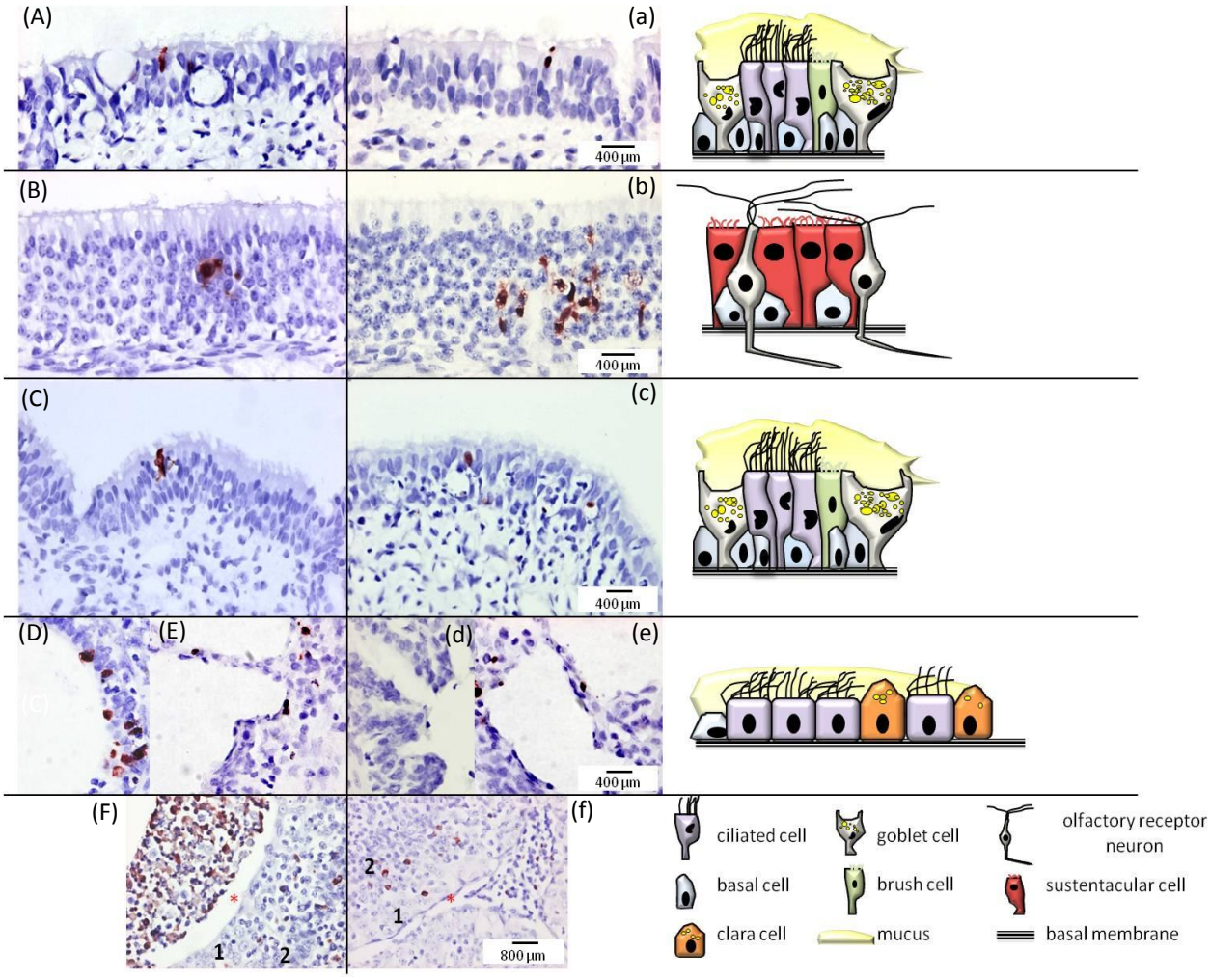

Figure 5: Immunohistochemical staining with HB65 against viral NP in infected animals. The left colomn shows R1-HK infected tissues, the middle colomn R2-HK infected tissues and the right colomn a schematic overview of the differenct cell types present in that particular epithelium. (A, a): nasal mucosa respiratory part, (B, b): nasal mucosa olfactory part, (C,c): trachea, (D, d): bronchioles, (E, e): alveolar tissues and (F, f): tonsils. In (F) and (f) the following structures are marked: lumen of the crypt filled with cellular debris (*), stratified squamous epithelium (1), diffuse lymphoid tissue (2). Most of the virus positive cells display a dark brown staining in the nucleus.

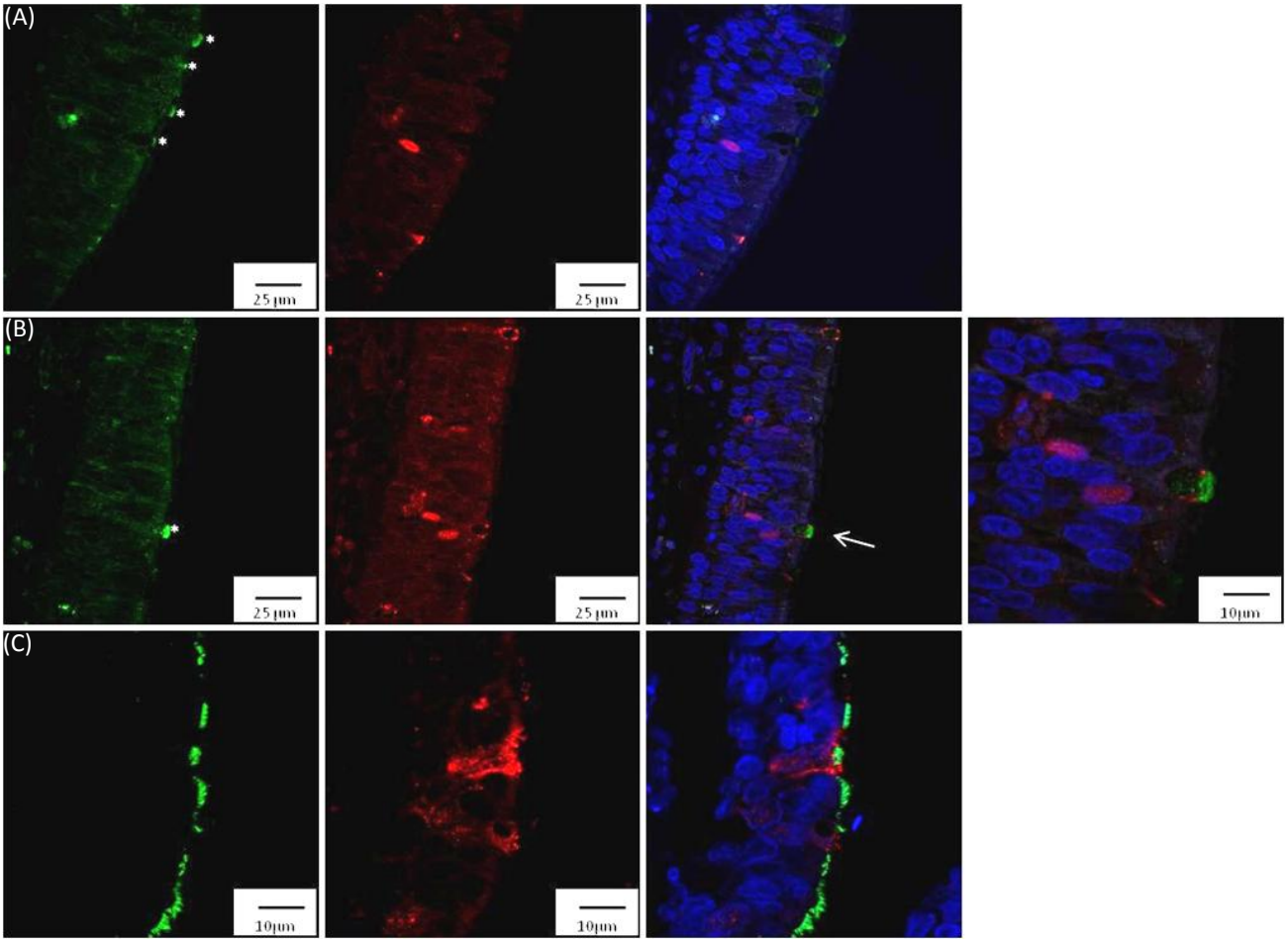

Figure 6: Infection of nasal explants by R1-HK as determined by immunostaining. Infected cells were stained with anti-nucleoprotein antibody (red), mucus producing cells (A + B) were stained using anti-mucin 5Ac (green) and ciliated cells (C) were stained using anti-beta tubulin antibody (also in green). The (*) represents individual goblet cells in the epithelial lining. The arrow indicates the region that is magnified in the next panel.

4.2.4.4. Infectivity reduction assays with mucus and porcine surfactant proteins

The inhibition assay to measure virus-mucus interactions, showed that R1-HK had significantly less remaining infectious cells than R2-HK regardless the mucus fraction used (blue lines in Figure 7). Comparing the % remaining infected cells after mucus incubation with the negative control, revealed R1-HK was significantly decreased under both conditions (red lines). For R2-HK this was only true after incubation with the sol layer (green line). Performing this test with two different doses of SP-D generated results similar to those after incubation with mucus. Once more the R1-HK was more strongly inhibited (in a dose dependent manner) than R2-HK. SP-A on the other hand seemed to exert an infection increasing influence at the highest dose used. This infection enhancing effect was strongest for R1-HK as visible by the statistical significant difference with the negative control (red line).

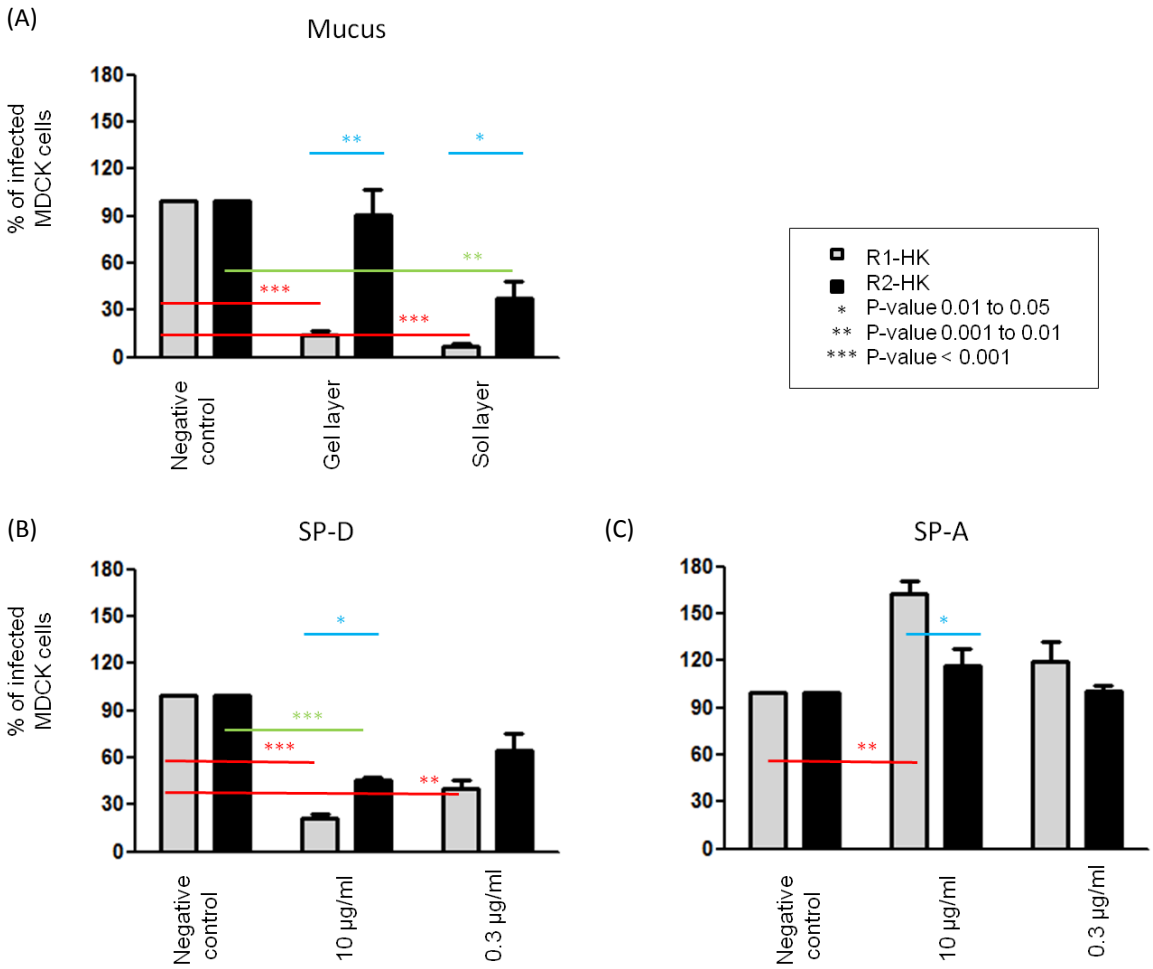

Figure 7: Infection reduction assay with mucus (A), recombinant porcine SP-D (B) and pSP-A (C). The reduction or increase in infectivity was expressed as the percentage of infected cells compared to a negative control which is set at 100%. The green lines indicate a significant difference between the % of R2-HK infected MDCK-cells under different conditions, the red lines indicate a significant difference between the % of R1-HK infected MDCK-cells under different conditions and the blue lines show significant differences between the % R1-HK and R2-HK infected cells under identical conditions.

4.2.5. Discussion

In the present study we characterized two viruses that differed only by receptor binding phenotype in terms of glycan binding properties, virus release capacities and interactions with non-specific antiviral barriers such as mucus and surfactant proteins *in vitro*. Additionally, the cell tropism of both viruses was assessed *in vivo*.

Firstly, two obvious explanations for the inefficient replication of R2-HK in pigs were proposed (Van Poucke et al. 2012). The two amino acid substitutions in the HA surface glycoprotein could result in a reduced incorporation of HA-molecules in the virion, a changed distribution of the HA-molecules on the virion surface or a disturbance in the balance of the HA-NA functions. The Western blot results, although not the most accurate protein quantification method (Heidebrecht et al. 2009), revealed no

obvious differences between R1-HK and R2-HK in the amounts of HA present on the virion surfaces. Even if a small distinction would be present, it is unlikely to be the cause of the pronounced phenotypic divergence observed between both viruses in pigs.

As HA and NA have antagonistic roles in the attachment and the release of virions, profound mutations in either glycoprotein can disrupt the balanced functions necessary for efficient virus replication (Kobasa et al. 1999, Mitnaul et al. 2000, Wagner et al. 2002, Su et al. 2009, Xu et al. 2012). The glycan microarray clearly showed a preferential binding of R2-HK to terminal Siaα2-3. As early Hong Kong/1/68 isolates were shown before to have a NA that acquired only a limited activity against α2-6 linkages and that maintained a predominant activity against α2-3 linkages (Baum and Paulson 1991, Kobasa et al. 1999), the NA activity by definition should thus be balanced. The results from our virus attach-release assay indicate that the R2-HK is not compromised in its release from MDCK-cells. Yet this assay is not a 100% conclusive as the catalytic activity of the NA is also influenced by the type of substrate. A study with viruses containing NA stalk deletions e.g. revealed no reduced cleavage from simple sialyloligosaccharides but did when the more complex fetuin was used as substrate (Castrucci and Kawaoka, 1993). Electron microscopy could be an alternative method to confirm an efficient HA-NA balance. Large virus aggregates attached to the cell membrane or floating in the supernatant should thus be absent (Liu et al. 1995, Sorrell et al. 2011). In addition, several research groups have shown that a reduced NA function also can be compensated by mutations in the HA that result in a lower binding affinity for (high molecular weight) Sia receptors (Kaverin et al. 1998, 2000; Mitnaul et al. 2000, Hughes et al. 2000, Gulati et al. 2009, Gen et al. 2012). The smaller fraction of R2-HK viruses attaching to the MDCK cells in combination with a faster elution of R2-HK compared to R1-HK in our test, might be supportive for a lower binding capacity of the R2-HK virus. Whether the overall binding capacity of R2-HK is really poorer than for R1-HK, could be tested in a microarray analysis given that equal amounts of viruses are loaded on the array. In the current test, hemagglutinating units (HAU)/ml, determined with turkey red blood cells, were used to standardize the virus quantity. Since it has been shown that turkey red blood cells carry more Siaα2-6 than Siaα2-3 (Medeiros et al. 2001, Neumann et al. 2009), R1-HK is probably hemagglutinating these cells more efficiently. As a consequence 1 HAU of R1-HK most likely contained fewer virus particles than 1 HAU of R2-HK. As such, the current array only permitted the comparison of the type of glycans bound by both viruses but not their binding affinity. In conclusion, the tests performed to characterize the recombinant virus together with the efficient replication of R2-HK in LE and certain pig tissues, do not indicate that this virus is intrinsically replication-incompetent.

The findings of the *in vivo* cell tropism study in pigs are compatible with an earlier report by De Vleeschauwer et al. (2009). These researchers determined virus titers and immunofluorescence scores of a LP avian H5N2 and swine H1N1 after intranasal or intratracheal inoculation of pigs. Similar to the R2-HK, they found that the avian H5N2 differed more from the swine H1N1 in its replication capacity rather than in their cell tropism. The H5N2 AIV infected proportionally fewer epithelial cells along the entire respiratory tract. In pig lung explants (Punyadarsaniya et al. 2011) ciliated cells were shown to be infected by porcine H3N2, avian H7N7 and avian H9N2 influenza viruses. At the same time the porcine H3N2 and avian H7N7 also infected goblet cells and even submucosal cells. It would appear from the current and previous results that under more natural situations and under conditions permitting multiple replication rounds, the earlier described differential cell tropism of avian and mammalian influenza viruses might be less pronounced *in vivo* (Matrosovich et al. 2004a; 2007, Ibricevic et al. 2006, Thompson et al. 2006, Wan and Perez 2007).

In the light of the viral antigen distribution of R1-HK and R2-HK in the pig, it needs to be emphasized that the interpretation of a positive immunohistochemical (IHC) signal requires consideration of whether such a cell is truly productively infected. The uptake of viral antigens by antigen presenting and phagocytotic cells could equally result in a positive staining. If the latter is true, the positive signal will be restricted to the cytoplasm without involvement of the nucleus (Jung et al. 2002). This seemed to be the case for multiple cells in our study, particularly those located in the tonsil and in certain regions of the submucosa. This observation could explain why our virus titers detected in the tonsils at 2 DPI with R1-HK and R2-HK were very low or negative respectively, while IHC positive cells were detected rather abundantly (Van Poucke et al. 2012). A sound differentiation between cellular uptake or active viral replication can be obtained by detection of positive stranded RNA (either by RT-PCR or in situ hybridization with anti-sense probes) (Korteweg and Gu 2008). Although several investigators reported the infection of human brain tissue with HPAIV H5N1 (Gu et al. 2007, Jang et al. 2009, Zhang et al. 2009), similar observations in pigs to our knowledge are restricted to one paper by De Vleeschauwer et al. (2009). They isolated both swine H1N1 (3/6 pigs) and chicken H5N2 (3/12 pigs) influenza virus from the brain stem. Considering the absence of viremia in pigs and the successful infection of receptor neurons in the olfactory part of the nasal mucosa during this experiment, virus trafficking via afferent neurons seems a plausible route of spreading. Even more because infection of the brain (De Vleeschauwer et al. 2009) was only seen after intranasal inoculation, not after intratracheal inoculation. Taken together, the switch in receptor binding preference of R2-HK did not result in an explicit switch of cell types infected by this virus in the porcine respiratory tract. Yet, the pronounced infection of the olfactory part of the nasal mucosa by the R2-Hk virus could have some pathogenic consequences.

Since the *in vivo* study did not reveal a distinct differentiation in cell tropism between both viruses but rather a quantitative difference, we focused on the virus interactions with possible other selective host factors. The fact that the R2-HK inoculated animals showed considerable individual variations in replication between pigs, points towards a possible role of innate factors other than receptor expression on epithelial cells. Numerous literature data on antiviral effects of airway mucins recognize that their carbohydrate chains are decorated with terminal Sia and that they attach to influenza viruses (Andrewes et al. 1954, Couceiro et al. 1993., Ehre et al. 2012). The way in which these mucins interact with different viruses, however, has been a topic of discussion. The traditional hypothesis is that mammalian hosts such as humans, pigs and ferrets are rather resistant to infections with AIV because the influenza virus gets trapped and eliminated in mucus carrying mostly terminal Siaα2-3 (so called decoy receptors) (Matrosovich and Klenk 2003). Nevertheless, lectin histochemistry results focusing on mucus, goblet cells and submucosal glands in all 3 species showed the presence of mainly Siaα2-6 residues (Kesimer et al. 2009, Nelli et al. 2010, Van Poucke et al. 2010, Punyadarsaniya 2011, Roberts et al. 2011, Jayaraman et al. 2012). Additionally, virus infection inhibition assays with nasal washes or exosomes, performed earlier by Roberts et al. (2011) and Kesimer et al. (2009) respectively, illustrated a strong(er) reduction of viruses with a Siaα2-6 binding receptor specificity. These results are indicative for a more solid binding of viruses with Siaα2-6 binding preferences to these components. This is in full accordance with our current findings where fewer infectious R1-HK virus particles remained, especially after incubation with the gel fraction of the porcine mucus. The results obtained in the SP-D assay followed a similar trend and substantiated the report by Hillaire et al. (2011). These researchers also observed a stronger infection reduction of human and swine H3N2 influenza isolates compared to avian strains. In addition to the Siaα2-6 present on porcine SP-D that can bind to the HA receptor binding site, this SP-D is also capable of interacting in a Ca^{2+} dependent manner with high mannose carbohydrates on the HA (van Eijk 2004, 2012). Since, even at the highest dose SP-D, the difference in inhibition between R1-HK and R2-HK was less pronounced than with mucus, we assume that both interaction ways took place. An attempt to quantify the importance of the Ca^{2+} dependent interaction by diluting the SP-D in Ca^{2+} and Mg^{2+} deprived DPBS was not successful because the MDCK cells were detaching. Why the SP-A interaction resulted in an infection enhancing effect, we have no indications at this point.

The above mucus and SP-D interactions seem paradoxical with the efficient replication of Siaα2-6 binding influenza viruses in humans, pigs and ferrets. We, therefore, hypothesize that the stronger binding of R1-HK compared to R2-HK to MDCK cells, mucus and SP-D reflects a facilitative capacity to penetrate this physical barrier *in vivo* by host-adapted viruses compared to AIVs and that this barrier

could be a more determining factor for the outcome of infection than strictly a difference in cell tropism.

4.2.6. Acknowledgements

The authors would like to express their gratitude to Keving Fung, Nele Dennequin, Carine Boone, Melanie Bauwens, Lieve Sys, Zeger Van den Abeele and Bart Ellebaut for excellent technical support. Thanks to Xiaoyun Yang for the fruitful mucus discussions.

4.2.7. References

Andrewes C.H., Isaacs A., Marmion B.P. (1954). Neutralising action of human nasal secretions on neutrotropic influenza virus. Baum L.G. and Paulson J.C. (1990). Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. Acta Histochem Suppl 40: 35-38.

Baum L.G. and Paulson J.C. (1991). The N2 neuraminidase of human influenza virus has acquired a substrate specificity complementary to the hemagglutinin receptor specificity. Virology 180: 10-15.

Castrucci M.R. and Kawaoaka Y. (1993). Biological Importance of Neuraminidase Stalk Length in Influenza A Virus. J Virol 67: 759-764.

Couceiro J.N.S.S., Paulson J.C., Baum L.G. (1993). Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res 29: 155-165.

Couceiro J.N.S.S. and Baum L.G. (1994). Characterization of the Hemagglutinin Receptor Specificity and Neuraminidase Substrate Specificity of Clinical Isolates of Human Influenza A Viruses. Mem Inst Oswaldo Cruz 89: 587-591.

De Vleeschauwer A., Atanasova K., Van Borm S., et al. (2009). Comparative Pathogenesis of an Avian H5N2 and a Swine H1N1 Influenza Virus in Pigs. PLoS ONE 4: doi:10.1371/journal.pone.0006662.

Ehre C., Worthington E.N., Liesman R.M. et al. (2012). Overexpressing mouse model demonstrates the protective role of Muc5ac in the lungs. PNAS www.pnas.org/cgi/doi/10.1073/pnas.1206552109

Gen F., Yamada S., Kato K. et al. (2012). Attenuation of an influenza A virus due to alteration of its hemagglutinin-neuraminidase functional balance in mice. Arch Virol doi: 10.1007/s00705-012-1577-3

Gu J., Xie Z., Gao Z. et al. (2007). H5N1 infection of the respiratory tract and beyond: a molecular pathology study. Lancet 370: 1137-1145.

Gulati S., Smith D.F., Air G.M. (2009). Deletions of neuraminidase and resistance to oseltamivir may be a consequence of restricted receptor specificity in recent H3N2 influenza viruses. Virol J 6: doi: 1.1186/1743-422X-6-22.

Haagsman H.P., Herias V., van Eijk M. (2003). Surfactant phospholipids and proteins in lung defence. Acta Pharmacol Sin 24: 1301-1303.

Heidebrecht F., Heidebrecht A., Schulz I. et al. (2009). Improved semiquantitatve Western blot technique with increased quantification range. J Immun Meth 345: 40-48.

Hillaire M.L.B., van Eijk M., van Trierum S.E. et al. (2011). Assessment of the Antiviral Properties of Recombinant Porcine SP-D against Various Influenza A Viruses In Vitro. PLoS ONE 6: doi:10.1371/journal.pone.0025005.

Hughes M.T., Matrosovich M., Rodgers M.E. et al. (2000). Influenza A viruses lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice. J Virol 74: 5206-5212.

Jang H., Boltz D., Sturm-Ramirez K. et al. (2009). Highly pathogenic H5N1 influenza virus can enter the central nervous system and induce neuroinflammation and neurodegeneration. PNAS 106: 11063-11068.

Jayaraman A., Chandrasekaran A., Viswanathan K. et al. (2012). Decoding the Distribution of Glycan Receptors for Human-Adapted Influenza A Viruses in Ferrets Respiratory Tract. PLoS ONE 7: doi:10.1371/journal.pone.0027517

Jung T., Choi C., Chae C. (2002). Localization of Swine Influenza Virus in Naturally Infected Pigs. Vet Path J 39: 10-16.

Kaverin N.V., Gambaryan A.S., Bovin N.V. et al. (1998). Postreassortment changes in influenza A virus hemagglutinin restoring HA-NA functional match. Virology 244: 315-321.

Kaverin N.V., Matrosovich M.N., Gambaryan A.S. et al. (2000). Intergenic HA-NA interactions in influenza A virus: postreassortment substitutions of charged amino acid in the hemagglutinin of different subtypes. Virus Res 66: 123-129.

Kesimer M., Scull M., Brighton B. et al. (2009). Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: a possible role in innate defence. FASEB J 23: 1858-1868.

Kobasa D., Kodihalli S., Luo M. et al. (1999). Amino Acid Residues Contributing to the Substrate Specificty of the Influenza A Virus Neuraminidase. J Virol 73: 6743-6751.

Korteweg C. and Gu J. (2008). Pathology, Molecular Biology, and Pathogenesis of Avian Influenza A (H5N1) Infection in Humans. Am J Pathol 172: 1155-1170.

Ibricevic A., Pekosz A., Walter M.J. et al. (2006). Influenza Virus Receptor Specificity and Cell Tropism in Mouse and Human Airway Epithelial Cells. J Virol 80: 7469-7480.

Liu C., Eichelberger M.C., Compans R.W. et al. (1995). Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. J Virol 69: 1099-1106.

Löndt B.Z., Brookes S.M., Nash B.J. et al. (2012). The infectivity of pandemic 2009 H1N1 and avian influenza viruses for pigs: an assessment by *ex vivo* respiratory tract organ culture. Influenza and other Resp Viruses doi: 10.1111/j.1750-2659.2012.00397.x

Matrosovich M. and Klenk H. (2003). Natural and synthetic sialic acid-containing inhibitors of influenza virus receptor binding. Rev Med Virol 13: 85-97.

Matrosovich M.N., Matrosovich T.Y., Gray T. et al. (2004a). Human and avian influenza viruses target differenct cell types in cultures of human airway epithelium. PNAS 101: 4620-4624.

Matrosovich M.N., Matrosovich T.Y., Gray T. et al. (2004b). Neuraminidase Is Important for the Initiation of Influenza Virus Infection in Human Airway Epithelium. J Virol 78: 12665-12667.

Matrosovich M., Matrosovich T., Garten W. et al. (2006). New low-viscosity overlay medium for viral plaque assays. Virology J doi:10.1186/1743-422X-3-63.

Matrosovich M., Matrosovich T., Uhlendorff J. et al. (2007). Avian-virus-like specificity of the hemagglutinin impedes influenza virus replication in cultures of human airway epithelium. Virology 361: 384-390.

Medeiros R., Escriou N., Naffakh N. et al. (2001). Hemagglutinin residues of recent human A(H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. Virology 289:74–85.

Mitnaul L.J., Matrosovich M.N., Castrucci M.R. et al. (2000). Balanced Hemagglutinin and Neuraminidase Activities Are Critical for Efficient Replication of Influenza A Virus. J Virol 74: 6015-6020.

Nelli R.K., Kuchipudi S.V., White G.A. et al. (2010) Comparative distribution of human and avian type sialic acid influenza receptors in the pig. Vet Res 6: doi:10.1186/1746-6148-6-4.

Neumann G., Noda T., Kawaoka Y. (2009). Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459:931–939.

Nobusawa E., Ishihara H., Morishita T. et al. (2000). Change in Receptor-Binding Specificity of Recent Human Influenza A Viruses (H3N2): A Single Amino Acid Change in Hemagglutinin Altered Its Recognition of Sialyloligosaccharides. Virology 278: 587-596.

Punyadarsaniya D., Liang C., Winter. et al. (2011). Infection of Differentiated Porcine Airway Epithelial Cells by Influenza Virus: Differential Susceptiblity to Infection by Porcine and Avian Viruses. PloS ONE 6: doi:10.1371/journal.pone.0028429.

Roberts K.L., Shelton H., Scull M. et al. (2011). Lack of transmission of a human influenza virus with avian receptor specificity between ferrets is not due to decreased virus shedding, but rather a lower infectivity in vivo. J Gen Virol 92: 1822-1831.

Sorrell E.M., Schrauwen E.J.A., Linster M. (2011). Predicting 'airborne' influenza viruses: (trans-) mission impossible? Current Opinion in Virology 1: 635-642.

Su B., Wurtzer S., Rameix-Welti M. et al. (2009). Enhancement of the Influenza A Hemagglutinin (HA)-Mediated Cell-Cell Fusion and Virus Entry by the Viral Neuraminidase (NA). PloS ONE 4: doi: 10.1371/journal.pone.0008495.

Thompson C.I., Barclay W.S., Zambon M.C. et al. (2006). Infection of Human Airway Epithelium by Human and Avian Strains of Influenza A Virus. J Virol 80: 8060-8068.

van Eijk M., van de Lest C.H.A., Batenburg J.J. et al. (2002). Porcine Surfactant Protein D Is N-glycosylated in its Carbohydrate Recognition Domain and Is Assembled into Differently Charged Oligomers. Am J Respir Cell Mol Biol 26: 739-747.

van Eijk M., White M. R., Batenburg J.J. et al. (2004). Interactions of Influenza A Virus with Sialic Acids Present on Porcine Surfactant Protein D. Am J Respir Cell Mol Biol 30: 871-879.

van Eijk M., van de lest C.H.A., White M. R. et al. (2012). Unique sugar-binding site mediates the distinct anti-influenza activity of pig surfactant protein D. J Biol Chem, 287: 26666-26677.

Van Poucke S.G.M., Nicholls J.M., Nauwynck H.J. et al. (2010). Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. Virol J 7: 38.

Van Poucke S., Uhlendorff J., Wang Z. et al. (2012). Effect of receptor specificity of A/Hong Kong/1/68 (H3N2) influenza virus variants on replication and transmission in pigs. Infl Other Resp Vir: doi: 10.1111/j.1750-2659.2012.00376.x

Wagner R., Matrosovich M., Klenk H. (2002). Functional balance between haemagglutinin and neuraminidase in influenza virus infection. Rev Med Virol 12: 159-166.

Wan H. and Perez D.R. (2007). Amino Acid 226 in the Hemagglutinin of H9N2 Influenza Viruses Determines Cell Tropism and Replication in Human Airway Epithelial Cells. J Virol 81: 5181-5191.

Xu R., Zhu X., McBride R. et al. (2012). Functional Balance of the Hemagglutinin and Neuraminidase Activities Accompanies the Emgergence of the 2009 H1N1 Inflluenza Pandemic. J Virol 86: 9221-9232.

Zhang Z., Zhang Z., Huang K. et al. (2009). Systemic infection of avian influenza A virus H5N1 subtype in humans. Hum Pathol 40: 735-739.

CHAPTER 5

Assessment of the contribution of hemagglutinin amino acids 62, 81, 92, 144 and 193 to the mammalian host adaptation of A/Hong Kong/1/68 (H3N2).

Van Poucke S.G., Baumann J., Qiu Y., Matrosovich M., Van Reeth K.

Manuscript in preparation

5.1. Abstract

The hemagglutinin glycoprotein (HA) of the 1968 Hong Kong pandemic influenza virus originated from an avian virus and differed from the putative avian precursor by seven amino acid substitutions (R62I, D81N, N92K, A144G, N193S, Q226L, and G228S). The Q226L and G228S mutations were shown to be essential for the HA adaptation to recognize epithelial receptors in the mammalian respiratory tract. To test whether the remaining five amino acid changes in the HA could have played a role in the emergence of the 1968 pandemic virus, we compared the replication ex vivo and the transmissibility in pigs of the recombinant pandemic virus A/Hong Kong/1/68 (H3N2) (R1-HK) and its HA mutant containing the five amino acid reversions back to the ancestral avian sequence (R5-HK).

Although R5-HK replicated efficiently in porcine respiratory explants and in donor pigs as measured by nasal virus shedding, the transmissibility of this virus was delayed and less effective compared to R1-HK. Sequence analysis of nasal swabs material from donor and contact animals often revealed the mutation D60G in the HA of contact pigs. This mutation was shown to affect the replication efficiency of the virus at changing pH.

The current results suggest that successful transmission of an influenza virus from an avian to mammalian host may require a combination of receptor-binding and additional changes in the HA. Similar observations were recently reported for the highly pathogenic avian H5N1 in a ferret model.

5.2. Introduction

The H3N2 1968 influenza pandemic was caused by a virus in which only the HA and PB1 of the prevalent human strain H2N2 were replaced by avian genes. Shortly after its transmission to the human population, seven mutations occurred in the HA1 glycoprotein (Bean et al. 1992, Ha et al. 2003). Two of these mutations, Q226L and to a lesser extent G228S, have been shown to be responsible for a switch in receptor binding properties from Siaα2-3 towards Siaα2-6 linkages in H2, H3, H4 and H9 influenza subtypes (Matrosovich et al. 2000, Wan and Perez 2007, Bateman et al. 2008, Scull et al. 2009). An enhanced Siaα2-6 binding affinity of influenza viruses was demonstrated to be a crucial prerequisite for efficient replication and transmission in several mammalian hosts including humans (Wilks et al. 2012), ferrets (Tumpey et al. 2007, Wan et al. 2008, Pappas et al. 2010, Roberts et al. 2011) and pigs (Van Poucke et al. 2012). Whether one or several of the 5 additional mutations in the A/Hong Kong/1/68 HA1: R62I, D81N, N92K, A144G and N193S were also essential for the adaptation to humans is not established.

Although an increasing number of avian H5N1 (Shinya et al. 2005, Yamada et al. 2006) and H9N2 (Matrosovich et al. 2001, Choi et al. 2004) influenza virus strains with (at least a partially) enhanced human-like receptor specificity have continuously been circulating in land-based poultry in Eurasian countries they have, so far, not evolved in a virus lineage with sustained transmissibility between humans or pigs (Peiris et al. 1999, Butt et al. 2005, Cong et al. 2007). These observations suggest that additional molecular or biological features, apart from the receptor binding properties of an influenza virus, might be critical for efficient transmission.

Wan et al. (2008) studied the direct contact transmissibility of three H9N2 influenza isolates with a human-like receptor specificity as a consequence of Q226L mutations in the HA. They found that only one of the viruses was transmitting to all ferrets. Similarly, Yen et al. (2007) examined the ferret transmissibility of four human H5N1 isolates, of which two isolates had an exclusive Siaα2-3 receptor specificity and two had an additional binding affinity for Siaα2-6 linkages. The only isolate which was inefficiently but consistently transmitted to contact ferrets possessed exclusive Siaα2-3 receptor specificity. These researchers concluded that an increased human-like receptor affinity did not automatically confer a higher transmission between ferrets which, analogous to humans and pigs, have a predominance of Siaα2-6 linkages in the respiratory tract (Leigh et al. 1995). Similarly, Maines et al. (2006) observed a lack of aerosol and direct contact transmission between ferrets for natural H5N1 avian influenza viruses (even when they were human isolates) and for reassortants of the latter virus with HA and NA from human A/Victoria/1/75 (H3N2). Only recently, Herfst et al. (2012) successfully created an avian H5N1 influenza virus that is transmissible in ferrets. They used a

combination of targeted mutagenesis in the HA and PB2 genes followed by serial passages in ferrets. Introducing Q226L and G228S mutations in the HA and an E627K mutation in PB2 resulted in a virus that was transmissible by direct contact but not by airborne contact. Additional H107Y and T160A substitutions in the HA were needed to obtain a virus with the potential to transmit by airborne contact. Phenotypically these extra substitutions resulted in an enhanced binding of Siaα2-6 linkages and a decreased affinity for Siaα2-3 linkages.

We, therefore, hypothesized that one or several of the 5 above mentioned mutations in the HA1 of A/Hong Kong/1/68 may have had a crucial contribution to breaching the species barrier and to establish virus transmissibility in mammals. This hypothesis was studied by comparing the replication in pigs and direct contact transmission among pigs of reverse genetics-generated variants with the avian-like residues in these positions (R5-HK) and the wild type sequence (R1-HK). Although R5-HK replication *ex vivo* and nasal virus shedding *in vivo* was not significantly different from that of the R1-HK, the transmission of R5-HK by direct contact between pigs was inefficient. Additionally, a natural mutant of R5-HK with an extra HA1 substitution, D60G, was isolated in several contact pigs. This mutation was located in the vestigial esterase domain of the HA1, a region known to interact with the B-loop of HA2. This interaction was earlier shown to be involved in the low pH dependent conformational change that influenza A viruses require for fusion of the envelope with the endosomal membrane (Ha et al. 2002). For that reason, the infectivity of all reverse genetics-generated viruses and the natural mutant was assessed under pH increasing conditions.

5.3. Materials and methods

5.3.1. Animals and viruses

Six- to 8-week-old pigs were obtained from a commercial herd serologically negative for influenza. All animals were tested for influenza virus antibodies before the start of the experiment by hemagglutination inhibition (HI) assay, by a competitive anti-influenza A nucleoprotein enzyme immunoassay (Idexx Laboratories) and by immunoperoxidase monolayer assay (IPMA) as described earlier. Pigs were housed in separate high-efficiency particulate air-filtered isolation units. At arrival they were treated intramuscularly with ceftiofur (Naxcel®, Pfizer-1ml/20 kg body weight). All experiments were authorized by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine, Ghent University.

The viruses R1-HK and R5-HK were generated using the eight plasmids reverse genetics system described by Hoffmann et al. (2000) and site-directed mutagenesis to introduce the amino acid substitutions. R1-HK harbors all eight original genes of the pandemic human virus A/Hong Kong/1/68 (H3N2) whereas R5-HK has the following HA substitutions: I62R, N81D, K92N, G144A and S193N.

Virus stocks were prepared in MDCK cells and their infectivity was determined by plaque titration in MDCK cells. From the nasal swab material of R5-HK contact pigs a virus with an additional mutation D60G was identified. This virus was called R5+1-HK. In the NH_4Cl infectivity test an extra recombinant virus, R7-HK, was included. This virus, created in a similar way as the R5-HK, contained all the avian precursor amino acids I62R, N81D, K92N, G144A, S193N, L226Q and S228G.

5.3.2. Porcine respiratory explants systems

Porcine nasal (NE), tracheal (TE), bronchial (BE) and lung (LE) explants were prepared as described in chapter 3. The replication potential of R1-HK and R5-HK inoculated at three different doses (10^4, 10^5 and 10^6 PFU) was assessed by determining the virus titers in supernatant at 1, 24 and 48 hours post inoculation. Virus titrations were performed on MDCK cells in 96-well plates followed by an immunocytochemical staining with monoclonal antibody HB-65 against viral nucleoprotein and virus titers were calculated by the method of Reed and Münch (1938).

5.3.3. Transmission studies

For each of the recombinant viruses, we used 6 directly inoculated (donor) pigs and 6 contact pigs. The 6 donor pigs of each group were housed in a separate isolation unit and inoculated intranasally with 10^6 PFU of R1-HK or R5-HK on day 0, as described in chapter 4.1. Two days after primary inoculation, 6 contact pigs were introduced into either the R1-HK or R2-HK inoculated group. The housing allowed both direct and airborne contact between inoculated and contact animals. Virus shedding was monitored by collecting nasal swabs from 0 through 9 days post inoculation (pi) or post contact (pc). Nasal swabs put into 1 ml of transport medium, were used for virus titration in MDCK cells and HA gene sequencing. Blood samples for serological examination were collected at 14 and 28 dpi/dpc. Transmission was defined by detection of virus from the nasal swabs and/or seroconversion in contact animals.

Antibody titers against the homologous viruses were determined by hemagglutination inhibition (HI), virus neutralisation (VN) and immunoperoxidase monolayer assay (IPMA). Antibodies against the viral NP were detected by a competitive anti-influenza A nucleoprotein enzyme immunoassay (NP-ELISA). HI, VN and IPMA assays were performed as described in chapter 4.1 (De Vleeschauwer et al 2010), the NP-ELISA antibody test (IDEXX Laboratories) was carried out following the manufacturer's instructions. Starting dilutions of the sera in the serological assays were 1:2 in VN, 1:5 in IPMA and 1:10 in HI and NP-ELISA. All sera were tested in duplicate. Antibody titers were expressed as the reciprocal of the highest serum dilutions that completely inhibited virus replication in MDCK cells (VN assay), that stained influenza infected MDCK cells (IPMA) and that inhibited hemagglutination (HI

assay). Results from the NP-ELISA were expressed as S/N ratio. The S/N response is the ratio of the sample optical density (OD) reading to the kit negative control OD reading.

5.3.4. Virus sequence analysis of HA genes

To monitor the molecular changes of R1-HK and R5-HK during transmission in pigs, HA gene sequence analysis was conducted on virus inoculum and on nasal swab samples collected from inoculated and contact pigs. Using the RNeasy Mini Kit (Qiagen), vRNA was extracted from 500 µl inoculum or nasal swab materials. This RNA was transcribed into cDNA with 1µl of Uni12 primer (-AGCCAAAAGCAGG-) and superscript III reverse transcriptase (In vitrogen) (Hoffmann et al. 2001). HA1 amplification was carried out using HA1 specific forward and reverse primers (-AGCAAAAGCAGGGGATAAT- and -AGTAGAAACAAGGGTGTTTTTA- respectively) and Herculase II fusion DNA polymerase (Agilent Technologies). RT-PCR products were excised from 2% agarose gels and purified using the Nucleospin gel and PCR clean-up kit from Macherey-Nagel.

5.3.5. Growth of R5+1-HK virus stock and phenotypic characterization

From the nasal swab material in which the D60G mutation was identified, we aimed to grow a new virus stock (referred to as R5+1-HK). This material was plaque-purified and 20 plaques were passaged on MDCK cells once and subsequently sequenced. After this one passage on MDCK cells, all of the grown viruses had reverted to the original R5-HK sequence, possibly because the selected plaques already contained a virus reverted to the R5-HK sequence. Alternatively, we grew virus stocks by directly inoculating the nasal swab material on MDCK cells. The HA sequence of these R5+1-HK stocks were confirmed and these viruses were used for further phenotypic characterization of the mutant.

Next, we assessed whether the additional HA-mutation could contribute to the pH of membrane fusion, a factor possibly affecting the efficiency of influenza virus transmission. An infection inhibition test with NH_4Cl was performed. MDCK-H cells in 96-well plates were infected with 100-200 PFU/100 µl virus in the presence of 0,25 mM NH_4Cl (stock: 100mM). NH_4Cl is a lysosomotropic agent known to accumulate in acidic cell compartments, such as the late endosome, and increases their pH. After infection the plates were incubated at 37 °C and 5% CO2 for 16 hours and afterwards fixed and stained immunohistochemically with monoclonal VS2525 against the influenza NP protein (kindly provided by Alexander Klimov CDC, USA). The percentage of remaining infectious viruses R1-HK, R2-HK, R7-HK, R5-HK and R5+1-HK in the presence of NH_4Cl were compared to an untreated infection. The assay was performed three times independently in three replicates each.

5.4. Results

5.4.1. *Ex vivo* replication of R1-HK and R5-HK

An overview of the virus replication results *ex vivo* is given in Figure 1. Assessing the replication potential of R1-HK and R5-HK in NE and TE showed no statistical significant difference between both viruses when an inoculation dose of 10^6 or 10^5 PFU was administered, although the average titers at 48 hpi were consistently higher for R1-HK than for R5-HK. At an inoculation dose of 10^4 PFU the difference between both viruses was more prominent, although still not statistically significant. Similar results were obtained in the BE, except that inoculation doses of 10^6 or 10^5 PFU resulted in indistinguishable virus titers at 48 hpi and that the overall virus yields were higher than in NE and TE. Virus yields in LE were, as expected from earlier results in chapter 3 and 4.1, identical between the two viruses regardless the inoculation doses that were used.

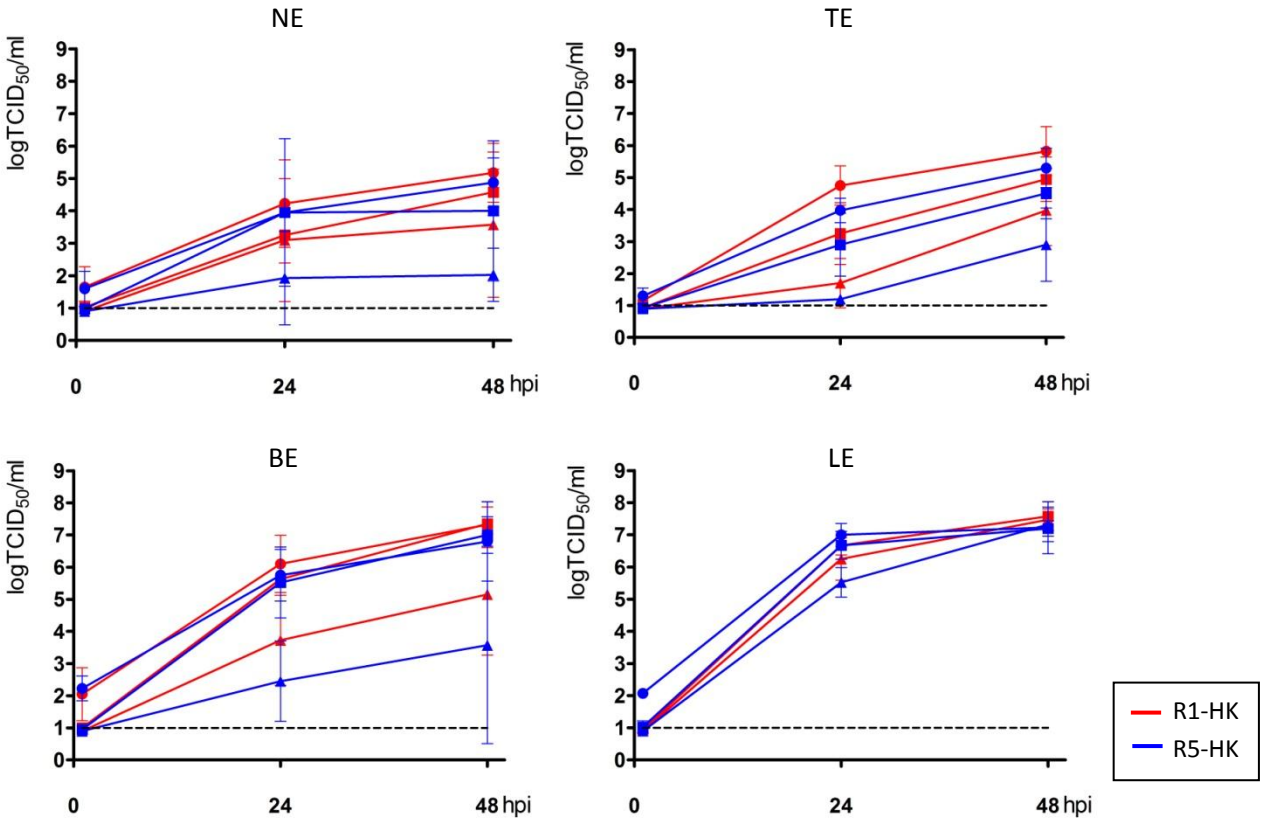

Figure 1: Virus yield in the supernatant of porcine nasal (NE), tracheal (TE), bronchial (BE) and lung explants (LE) of R1-HK and R5-HK. Virus titers are determined at 1, 24 and 48 hours post inoculation (hpi). Three different inoculation doses were assessed for each virus: 10^4 (triangles), 10^5 (squares) and 10^6 PFU (circles). Each point shows the mean and standard deviation of 4 repeats, the dashed line is the detection limit.

5.4.2. Transmission in pigs

No significant differences between the AUC's of R1-HK and R5-HK intranasally inoculated pigs were observed. Although virus was excreted by all 6 inoculated animals over a period of five to six days, distinctions between R1-HK and R5-HK nasal shedding were noticed at day 1 and 6 post inoculation, depicted in Figure 2 (A) and 2 (B) respectively. While R1-HK was immediately reaching peak titers between 5 and 6,5 $logTCID_{50}$/ml, R5-HK inoculated pigs needed until day 2 post inoculation to attain virus titers as high as for R1-HK. At 6 days post inoculation, 3 out of 6 R1-HK inoculated pigs still reached 4 $logTCID_{50}$/ml while all R5-HK pigs at this time had titers below 2 $logTCID_{50}$/ml. Transmission of R1-HK to the contact pigs occurred in all animals, detected by virus shedding in nasal swabs as early as the first day post contact in some pigs. Excretion in contact animals lasted for 7 to 8 days reaching peak titers similar to those observed in the inoculated group but with a somewhat slower starting profile. The AUC of the R1-HK inoculated pigs was not significantly different from the R1-HK contact pigs. In contrast, the transmission of R5-HK was highly inefficient. Only five out of six contact pigs were shedding virus at some point. Additionally, the transmission was strongly delayed, with the first positive nasal virus excretion detected at 6 dpc. At that moment (8 dpi) inoculated pigs were not even shedding the R5-HK virus any more. Although positive virus titers were still detected in 4 out of 6 pigs at 9 dpc, 3 pigs had already clearly reduced titers compared to the previous day. Based on this observation, we assume that virus excretion would not take place much longer if the titers were monitored over a longer time period. Compared to R1-HK contact pigs which shed virus for up to 8 days, R5-HK shedding occured during a shorter time period (4 to 5 days). Out of the 5 infected contact pigs, only 2 pigs (contact pigs 4 and 6) were reaching peak titers similar to the ones of the inoculated animals and this only during 1 day.

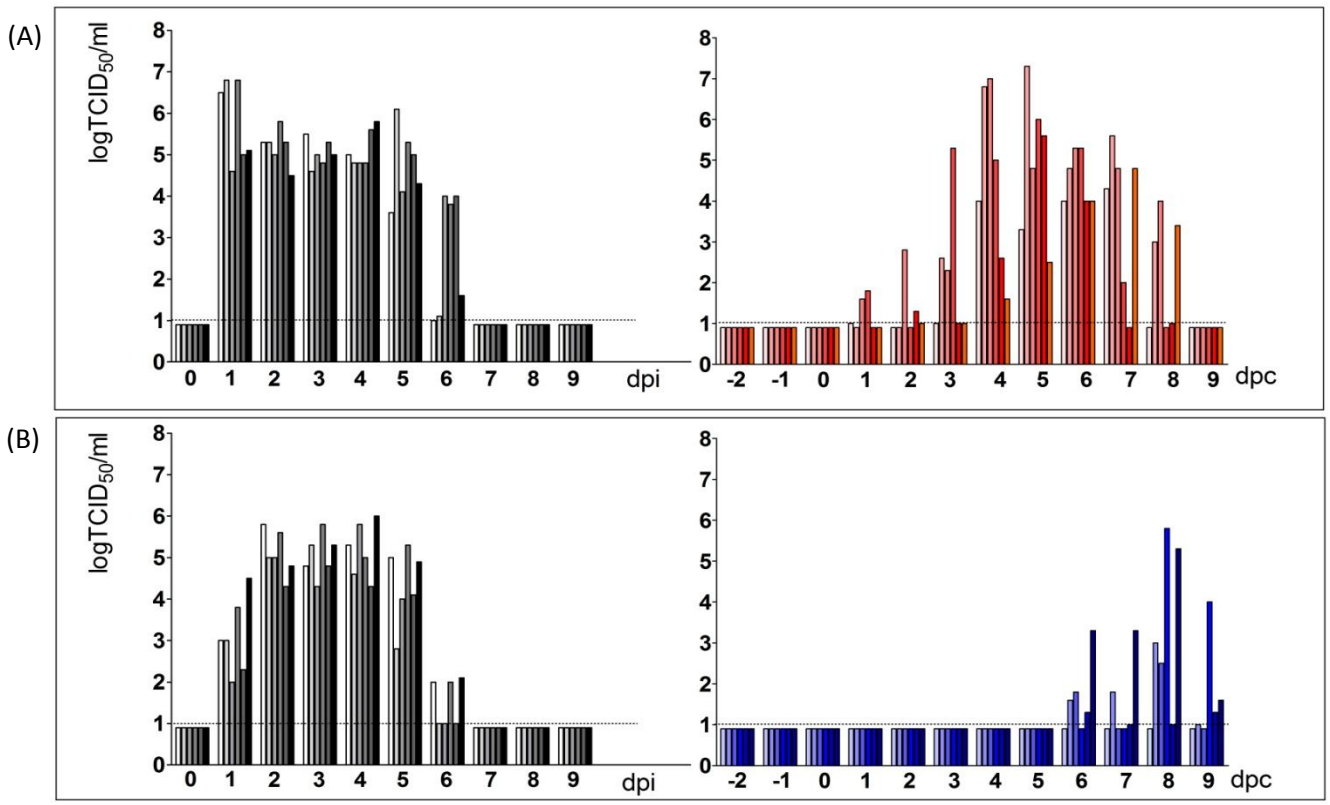

Figure 2: Virus titers of R1-HK (A) or R5-HK (B) in nasal swabs of individual pigs during 9 subsequent days post inoculation (dpi) or days post contact (dpc). The contact animals were introduced to the inoculated pigs at 2 dpi (equal to 0 dpc). Each bar represents the nasal shedding of one pig. The grey shades are inoculated pigs, the red shades R1-HK contact pigs and the blue shades R5-HK contact pigs. The dotted line represents the detection limit.

5.4.3. Serology

Four different assays were performed to determine the serological responses in inoculated and contact pigs at 14 and 28 days pi or pc. An overview of all individual results, for every time point and animal performed in duplicate, is given in Figure 3. All pigs inoculated with R1-HK or R5-HK viruses seroconverted by 28 dpi, but the average values obtained for R5-HK pigs in the SN-test, IPMA-test and NP-ELISA were slightly higher than for R1-HK inoculated pigs. In the HI-test this difference was not observed. By 14 dpi the overall levels of antibody detection were already somewhat higher for the R5-HK inoculated pigs, in particularly in the NP-ELISA where the majority of the R1-HK inoculated pigs were still negative at this time point. The serological responses for the pigs in the R1-HK contact group confirmed the results from the nasal virus shedding. All these animals, although not necessarily in all conducted tests, were positive. This was also true for the pigs in the R5-HK contact group although the virus titers in the nasal swabs of contact pig 1 (CP1) were below the detection limit during the entire monitored period. This pig had positive results in the SN- and IPMA-assay,

while another animal, CP5, with only limited virus excretion on day 6 and 9 pc was exclusively positive in the NP-ELISA.

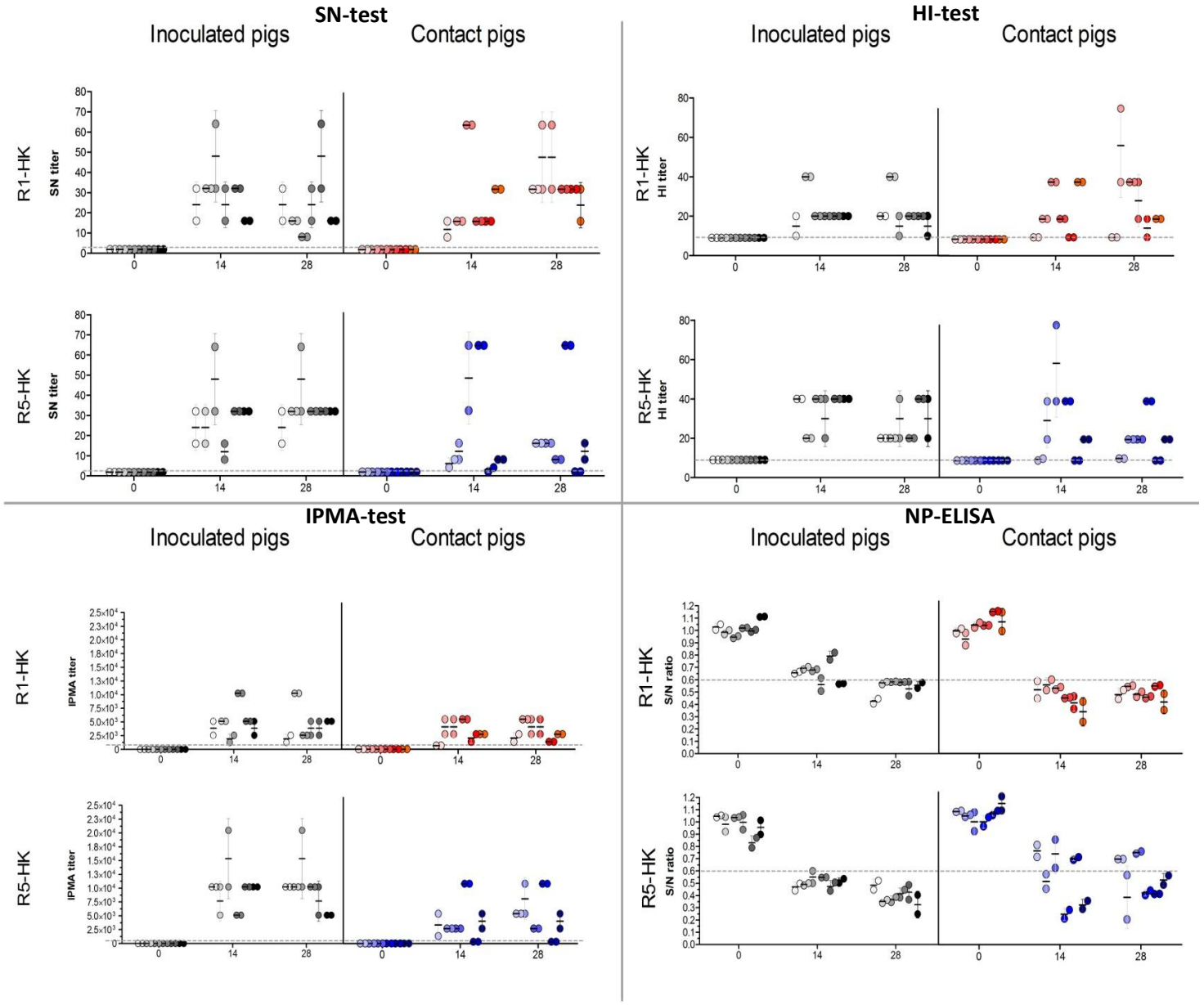

Figure 3: Antibody responses induced in individual pigs as determined by SN-test, HI-test, IPMA-test or NP-ELISA after inoculation (left panels) or contact (right panels) with R1-HK or R5-HK. Every test was performed in duplicate, as represented by two dots per pig. The short line represents the average of the repeats. The antibody titers are expressed as the reciprocal of the serum dilutions for VN, IPMA and HI assays and as S/N ratio for the NP-ELISA. S/N ratios ≥ 0,6 are considered negative. The grey dashed line is the detection limit for every test.

5.4.4. Molecular changes during the transmission of R5-HK

R1-HK viruses isolated from nasal swabs of all inoculated and contact pigs at 5 dpi or dpc respectively were used for HA sequencing. These viruses did not show mutations when compared with the HA sequence of A/Hong Kong/1/68 in GenBank (AF348176) and the HA in the inoculum.

Comparing the HA sequence of the R5-HK inoculum with the GenBank sequence AF348176 revealed the expected mutations I62R, N81D, K92N, G114A and S193N and one additional silent mutation at amino acid 273. Nasal swab samples from R5-HK inoculated pigs were used for RNA extraction at 4 and 6 dpi, provided that virus was detected at these time points. At 4 dpi this included all animals, at 6 dpi this was limited to pigs 1, 4 and 6. Analyses of these samples confirmed a sequence identical to the inoculum. However, four out of five contact pigs contained one mutation additional to the ones present in the inoculated pigs. The T206I mutation of contact pig 2 at 6 dpc was a single event. This location is in contact with the other HA's in a trimer conformation. In contact pigs 4, 5 and 6 another substitution was noticed: D60G, in close vicinity to the I62R mutation. Pigs 4 and 6 already contained this mutation at the earliest moment of detectable virus shedding while pig 5 only started to carry this mutation from day 2 of nasal virus excretion on. An overview of the mutations found in the different contact pigs at varying time points is shown in Figure 4A. In general, the contact pigs with the highest virus titers contained the D60G mutation. This is shown in Figure 4B.

(A)

	6 dpc		7 dpc		8 dpc		9 dpc	
	aa 60	aa 206	aa 60	aa 206	aa 60	aa 206	aa 60	aa 206
Contact pig 2	D	I			D	I	D	I
Contact pig 3	D	T			D	T		
Contact pig 4					G	T	G	T
Contact pig 5	D	T	D/G	T	G	T	G	T
Contact pig 6	G	T	G	T	G	T	G	T

(B)

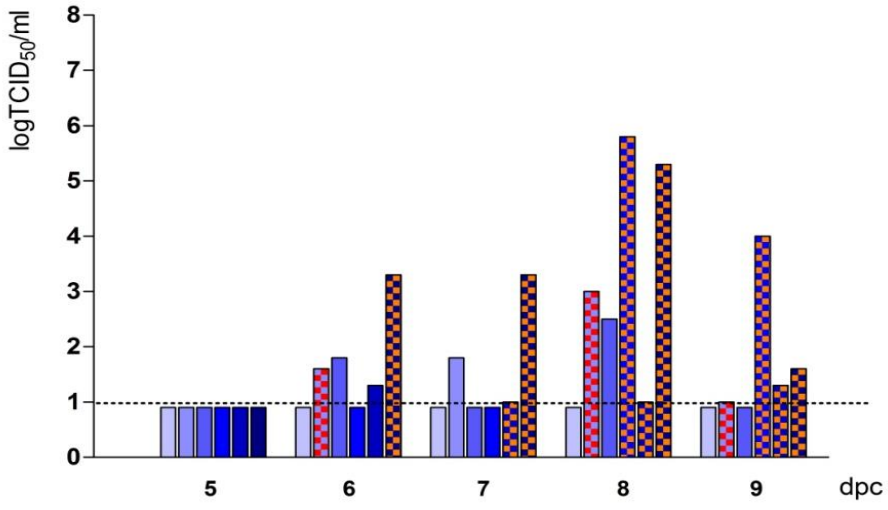

Figure 4: (A) Gives an overview of the amino acid (aa) residues 60 and 206 in the HA1 of R5-HK contact pigs at different time points after introduction in the group of inoculated animals. The grey boxes show no sequencing was performed, mostly because these pigs were not shedding virus at the indicated time points. Contact pig 3 contained the sequences of the parent virus. Red boxes refer to a mutation at amino acid 206, the orange boxes to a mutation at amino acid 60. (B) depicts the correlation between viral titers excreted by the individual

pigs from day 5 until 9 post contact and the presence of additional mutations in the HA1 of R5-HK. When substitutions were detected in the HA1, the bar has red (T206I) or orange (D60G) checkers.

5.4.5. NH_4Cl infectivity test

Since the above observed mutation, D60G, located to a region that has been shown before to be involved in the pH-induced conformational changes in the HA and the release of the viral genome in the target cell, we decided to investigate the virus infectivity under changed pH conditions. The percentages of remaining infected cells after incubation of the different viruses with 0,25 mM NH_4Cl, a lysosomotropic agent increasing the pH of intracellular vesicles, are shown in Figure 5. This assay revealed that the 226 and 228 mutations (R2-HK), although strictly located in the RBS, caused the virus to be significantly less infectious compared to R5-HK and R7-HK when the pH increased. The percentage of remaining infectious viruses of R5+1 were significantly lower compared to R1-HK, R5-HK and R7-HK.

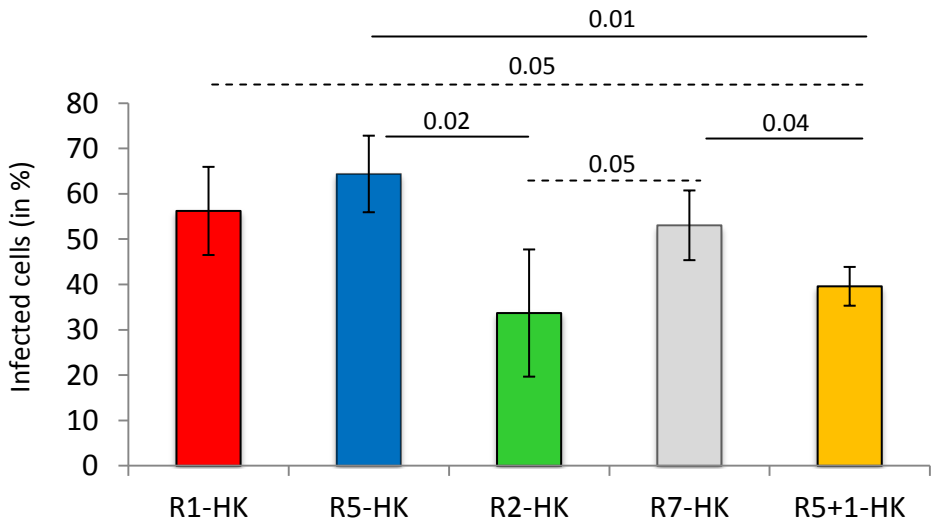

Figure 5: The percentage remaining infected cells after incubation of the virus with 0,25 mM NH_4Cl compared to a negative control. Each bar is the result of 3 repetitions, bars represent means ± standard deviations. All results were compared in a t-test, the p-values lower or equal to 0.05 are shown above the bars. A full line represents a p-value between 0.01 – 0.04 and a dotted line represents a p-value equal to 0.05.

5.5. Discussion

Transmission of avian influenza viruses to (and between) a mammalian host requires the bridging of a strong species barrier. Numerous researchers have reported a lack of transmission of avian influenza viruses to pigs (Shortridge et al. 1998, Loeffen et al. 2004, Choi et al. 2005, De Vleeschauwer et al. 2009) and ferrets (Herfst et al. 2012, Imai et al. 2012) under experimental conditions. Surveillance studies in the field indicated that exposure to high viral loads is demanded to obtain infection of humans (Beigel et al. 2005) and pigs (Loeffen et al. 2004, Choi et al. 2005) with

avian influenza viruses. Most reported cases of human infections with HP H5N1 or H7N7 influenza viruses revealed a history of direct contact with sick or dead poultry (Mounts et al. 1999, Bridges et al. 2002, Koopmans et al. 2004, Hayden and Croisier 2005). Avian influenza viruses that acquire Sia-binding properties similar to human viruses are supposed to have an increased capacity to transmit to mammals by respiratory droplets or direct contact (Tumpey et al. 2007, Wan et al. 2008, Sorrell et al. 2009, Pappas et al. 2010, Jayaraman et al. 2011, Van Poucke et al. 2012). Yet, a variety of viruses possessing an enhanced Siaα2-6 specificity still lack the capacity to transmit between mammals, indicating that additional biological viral features may play a role in the transmissibility of influenza viruses (Maines et al. 2006, Yen et al. 2007, Belser et al. 2008, Wan et al. 2008, Herfst et al. 2012, Imai et al. 2012). The purpose of this study was to examine whether additional mutations in the HA1 of an influenza virus, apart from those changing the receptor specificity from avian- to human-like, can contribute to the virus transmissibility to mammals. We, therefore, chose to focus on HA1 residues of pandemic A/Hong Kong/1/68 (H3N2) that changed shortly after its introduction into the human population being: R62I, D81N, N92K, A144G and N193S. Using reverse genetics, R1-HK wild-type virus and R5-HK harboring the 5 avian-like amino acids were constructed and studied for their direct contact transmission between pigs.

The transmission of R5-HK was inefficient and clearly delayed compared to R1-HK despite high viral nasal titers in all inoculated animals during 4 to 5 days, providing indications that one or several of the 5 substituted amino acids confer the transmissibility of this virus to pigs. In contrast with the earlier studied R2-HK, the area under the curve of the R5-HK inoculated pigs was not significantly lower compared to the wild-type virus (Van Poucke et al. 2012). These findings confirmed the virus replication results obtained in porcine respiratory explants, where differences between R5-HK and R1-HK in nasal, tracheal and bronchial tissues were minimal and non existing in lung tissues at inoculation doses of 10^6 and 10^5 PFU. Only at day 1 post inoculation, the pigs inoculated with R5-HK shed lower titers, indicating that this virus may be somewhat slower in replication. This observation is unlikely to explain the inefficient transmission of R5-HK, as the contact pigs were not introduced until day 2 post inoculation. A lack of transmission, despite high amounts of virus shed by infected animals, has been repeatedly described in ferrets for different avian influenza subtypes. Although high titers of infectious H5N1 AIV were detected in the nasal washes of these animals, several research groups observed no or inefficient transmission to direct contact ferrets (Maines et al. 2006, Yen et al. 2007). Even the pandemic H1N1, despite causing increased morbidity, higher replication titers in the lungs and presence in the intestinal tract in ferret, displayed a less efficient respiratory

droplet transmissibility in these animals compared to a human seasonal H1N1 (Maines et al. 2009, Jayaraman et al. 2011).

The first moment of nasal R5-HK detection in 4 out of 6 contact pigs corresponded with a time point of 8 dpi, when virus was no longer detected in nasal swabs of inoculated pigs. This observation raises questions regarding the mode of transmission of R5-HK. One possibility is that the inoculated pigs were still shedding virus at the moment of transmission, but to a level below the detection limit of our virus titration assay. This could also explain why one of the contact pigs, that never had detectable virus in nasal swabs, seroconverted. Lack of virus excretion in the presence of detectable antibodies was observed earlier in transmission experiments with avian influenza viruses in pigs and ferrets (De Vleeschauwer et al. 2009). Another option is that the R5-HK virus persisted in the environment over a longer time-period, permitting postponed transmission via fine droplets or contact transmission via surfaces (Weber and Stilianakis 2008). Since the experimental set-up permitted close interactions between inoculated and contact pigs during the monitored time laps, it is impossible to differentiate which transmission pathway occurred.

Sequencing of the HA1 in nasal swabs of R5-HK inoculated and contact pigs revealed the presence of one mutation in several contact pigs: D60G. Considering the close spatial proximity with amino acid 62 we postulate that this extra mutation might be a compensation for the I62R substitution, especially because the latter is introduced by 2 nucleotide changes to prevent reversion to the human-like residue. The D60G substitution is located in the vestigial esterase sub-domain together with the I62R, N81D and K92N. This domain was previously shown to modulate the virus stability in the environment as well as the pH-dependent fusion activity of HA by electrostatic interactions with the B-loop (Rachakonda et al. 2007, Xu and Wilson 2011). Only when the B-loop is unclamped by protonation of positively charged amino acids, a loop-to-helix transition which extends the central coiled-coil and causes a relocation of the fusion peptide towards the target membrane, can occur. Where the R5 and R5+1 mutations are located in respect to the different subdomains is shown in Figure 6. Both environmental stability and pH fusion activation have been shown to influence the host specificity, the pathogenicity and the transmissibility of an influenza virus. Comparing H7N3 isolates from turkeys and ducks revealed few amino acid differences in the HA, related with a higher fusion pH of the duck viruses to avoid inactivation in acidic tissues, such as the intestinal tract (Giannechini et al 2006). DuBois et al. (2011) demonstrated that an increased HA activation pH correlated with an increased H5N1 virulence in chickens, while Reed et al. (2010) observed an increased pathogenicity of another H5N1 AIV in ducks when the HA fusion pH decreased. A very recent study by Imai et al. (2012) for the first time generated indications that sustained transmission

between ferrets of an H5 reassortant virus (composed of the HA from HP H5N1 and remaining genes from pandemic H1N1) with human-like receptor binding properties may require a certain level of stability of the HA protein. In their initial experiment they obtained airborne transmission in 2 out of 6 contact ferrets. One virus, isolated from a contact ferret with high viral titers, was subsequently used in a second transmission experiment. In this study they obtained transmission to 4 out of 6 contact animals and sequencing of this virus revealed an extra T318I mutation in the HA. To explore the functional role of this mutation, they assessed HA-induced polykarion formation of HeLa cells at different pH and the heat stability of the virus. These researchers concluded that the mutation reduced the pH-threshold for fusion activity and increased the heat stability.

The research by Imai and co-workers (2012) displayed pronounced similarities with our current experiment in pigs. Firstly, the R5-HK resembles their H5 reassortant virus in the sense that the virus also carried a HA from avian origin with enhanced affinity for human-like Sia receptors. Secondly, the transmission of R5-HK was inefficient although we observed nasal shedding in 5/6 contact pigs whereas they observed it in 2/6 contact ferrets. This could be related to our less restrictive transmission model which, in addition to airborne transmission, permits direct contact as well. Finally, we moreover isolated a virus in the contact animals with high virus titers that contained an additional mutation which influences pH stability. Although the location of the mutation within the HA is different as well as the assays we used to evaluate pH-influences, we provided indications enforcing the idea that pH-stability and fusion activation of HA may be essential contributors to efficient transmission in mammals. Further transmission experiments with R5+1-HK in pigs as well as functional fusion tests *in vitro* such as those by Su et al. (2009) are needed to confirm this hypothesis and to unravel the exact mechanism.

5.6. Acknowledgements

The authors would like to acknowledge Lieve Sys, Nele Dennequin, Carine Boone, Melanie Bauwens, Zeger Van den Abeele and Bart Ellebaut for excellent technical support.

5.7. References

Bateman A.C., Busch M.G., Karasin A.I. et al. (2008). Amino acid 226 in the hemagglutinin of H4N6 influenza virus determines binding affinity for alpha2,6-linked sialic acid and infectivity levels in primary swine and human respiratory epithelial cells. J Virol 82: 8204-8209.

Bean W.J., Schell M., Katz J. et al. (1992). Evolution of the H3 Influenza Virus Hemagglutinin from Human and Nonhuman Hosts. J Virol 66: 1129-1138.

Beigel J.H., Farrar J., Han A. M. et al. (2005). Avian Influenza A (H5N1) Infection in Humans. N Engl J Med 353: 1374-1385.

Bridges C.B., Lim W., Hu-Primmer J. et al. (2002). Risk of influenza A (H5N1) infection among poultry workers, Hong Kong, 1997-1998. J Inf Dis 185: 1005-1010.

Belser J.A., Blixt O., Chen L.M. et al. (2008). Contemporary North American influenza H7 viruses possess human receptor specificity: Implications for virus transmissibility. PNAS 105: 7558-7563.

Butt K.M., Smith G.J., Chen H. et al. (2005). Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol 43: 5760-5767.

Choi Y.K., Ozaki H., Webby R.J. et al. (2004). Continuing evolution of H9N2 influenza viruses in Southeastern China. J Virol 78: 8609-8614.

Choi Y.K., Nguyen T.D., Ozaki H. et al. (2005). Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand. J Virol 79: 10821-10825.

Cong Y.L., Pu J., Liu Q.F. et al. (2007). Antigenic and genetic characterization of H9N2 swine influenza viruses in China. J Gen Virol 88: 2035-2041.

De Vleeschauwer A., Van Poucke S., Braeckmans D. ret al. (2009). Efficient transmission of swine-adapted but not wholly avian influenza viruses between pigs and from pigs to ferrets. J Infect Dis 200:1884-1892.

DuBois R.M., Zaraket H., Reddivari M. et al. (2011). Acid Stability of the Hemagglutinin Protein Regulates H5N1 Influenza Virus Pathogenicity. PLoS Pathogens 7: doi:10.1371/journal.ppat.1002398.

Hayden F. and Croisier A. (2005). Transmission of Avian Influenza Viruses to and between Humans. J Inf Dis 192:

Ha Y., Stevens D.J., Skehel J.J. et al. (2002). H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. EMBO J 21: 865-875.

Ha Y., Stevens D.J., Skehel J.J. et al. (2003). X-ray structure of the hemagglutinin of a potential H3 avian progenitor of the 1968 Hong Kong pandemic influenza virus. Virology 309: 209-218.

Herfst S., Schrauwen E.J.A., Linster M. et al. (2012). Airborne Transmission of Influenza A/H5N1 Virus Between Ferrets. Science 336: 1534-1541.

Hoffmann E., Neumann G., Kawaoka Y. et al. (2000). A DNA transfection system for the generation of influenza A virus from eight plasmids. PNAS 97: 6108-6113.

Hoffmann E., Stech J., Guan, Y. et al. (2001). Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146: 2275-2289.

Giannecchini S., Campitelli L., Calzoletti L. et al. (2006). Comparison of in vitro replicatio features of H7N3 influenza viruses from wild ducks and turkeys: potential implications for interspecies transmission. J Gen Virol 87: 171-175.

Imai M., Watanabe T., Hatta M. et al. (2012). Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 486: doi:10.1038/:nature10831.

Jayaraman A., Pappas C., Raman R. et al. (2011) A Single Base-Pair Change in 2009 H1N1 Hemagglutinin Increases Human Receptor Affinity and Leads to Efficient Airborne Viral Transmission in Ferrets. PLoS ONE 6(3): e17616. doi:10.1371/journal.pone.0017616

Koopmans M., Wilbrink B., Conyn M. et al. (2004). Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. The Lancet 363: 587-593.

Leigh M.W., Connor R.J., Kelm S. et al. (1995). Receptor specificity of influenza virus influences severity of illness in ferrets. Vaccine 13: 1468-1473.

Loeffen W., de Boer E., Koch G. (2004). Transmission of a highly pathogenic avian influenza virus to swine in the Netherlands. Proceedings: International Society for Animal Hygiene Saint Malo-France.

Maines T.R., Chen M.L., Matsuoka Y. et al. (2006). Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. PNAS 103: 12121-12126.

Maines T.R., Jayaraman A., Belser J.A. et al. (2009). Transmission and Pathogenesis of Swine-Origin 2009 A(H1N1) Influenza Viruses in Ferrets and Mice. Science 325: 484-487.

Matrosovich M., Tuzikov A., Bovin N. et al. (2000). Early alterations of the receptor-binding properties of H1, H2 and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol 74: 8502-8512.

Matrosovich M.N., Krauss S., Webster R.G. (2001). H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology 281: 156-162.

Mounts A.W., Kwong H., Izurieta H.S. et al. (1999). Case-control study of risk factors for avian influenza A (H5N1) disease, Hong Kong, 1997. J Inf Dis 180: 505-508.

Pappas C., Viswanathan K., Chandrasekaran A. et al. (2010). Receptor Specificity and Transmission of H2N2 Subtype Viruses Isolated from the Pandemic of 1957. PLoS ONE b5: doi:10.1371/journal.pone.0011158.

Peiris M., Yuen K.Y., Leung C.W. et al. (1999). Human infection with influenza H9N2. The Lancet 354: 916-917.

Rachakonda S.P., Veit M., Korte T. et al. (2007). The relevance of salt bridges for the stability of the influenza virus hemagglutinin. FASEB J 21: 995-1002.

Reed L.J. and Münch H. (1938). A simple method of estimating fifty per cent endpoints. Am J Hygiene 27: 493-497.

Reed M.L., Bridges O.A., Seiler P. et al. (2010). The pH of Activation of the Hemagglutinin Protein Regulates H5N1 Influenza Virus Pathogenicity and Transmissibilty in Ducks. J Virol 84: 1527-1535.

Roberts K.L., Shelton H., Scull M. et al. (2011). Lack of transmission of a human influenza virus with avian receptor specificity between ferrets is not due to decreased virus shedding, but rather a lower infectivity in vivo. J Gen Virol 92: 1822-1831.

Scholtissek C. (1985). Stability of infectious influenza A viruses at low pH and at elevated temperature. Vaccine 3: 215-219.

Scull M.A., Gillim-Ross L., Santos C. et al. (2009). Avian influenza virus glycoproteins restrict virus replication and spread through human airway epithelium at temperatures of the proximal airways. PLoS Pathogens 5: doi:10.1371/journal.ppat.1000424.

Shinya K., Hatta M., Yamada S. et al. (2005). Characterization of a human H5N1 influzna A virus isolated in 2003. J Virol 79: 9936-9932.

Shortridge K.F., Zhou N.N., Guan Y. et al. (1998). Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 252: 331-342.

Sorrell E.M., Wan H., Araya Y. et al. (2009). Minimal molecular constraints for respiratory droplet transmission of an avian-human H9N2 influenza A virus. PNAS 106: 7565-7570.

Stevens J., Blixt O., Glaser L. et al. (2006). Glycan Microarray Analysis of the Hemagglutinins from Modern and Pandemic Influenza Viruses Reveals Different Receptor Specificities. J Mol Biol 355: 1143-1155.

Su B., Wurtzer S., Rameix-Welti M. et al. (2009). Enhancement of the Influenza A Hemagglutinin (HA)-Mediated Cell-Cell Fusion and Virus Entry by the Viral Neuraminidase (NA). PLoS ONE 4: doi: 10.1371/journal.pone.0008495.

Tumpey T.M., Maines T.R., Van Hoeven N. et al. (2007). A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science: doi:10.1126/science.1136212

Van Poucke S., Uhlendorff J., Wang Z. et al. (2012). Effect of receptor specificity of A/Hong Kong/1/68 (H3N2) influenza virus variants on replication and transmission in pigs. Infl Other Resp Vir: doi: 10.1111/j.1750-2659.2012.00376.x

Wan H. and Perez D.R. (2007). Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human airway epithelial cells. J Virol 81: 5181-5191.

Wan H., Sorrell E.M., Song H. et al. (2008). Replication and Transmission of H9N2 Influenza Viruses in Ferrets: Evaluation of Pandemic Potential. PLoS ONE 3: doi: 10.1371/journal.pone.0002923.

Weber T.P. and Stilianakis N. (2008). Inactivation of influenza A viruses in the environment and modes of transmission: A critical review. J Inf 57: 361-373.

Wilks S., de Graaf M., Smith D.J. et al. (2012). A review of influenza hemagglutinin receptor as it relates to pandemic properties. Vaccine 30: 4369-4376.

Xu R. and Wilson I.A. (2011). Structural Characterization of an Early Fusion Intermediate of Influenza Virus Hemagglutinin. J Virol 85: 5172-5182.

Xu Q., Wang W., Cheng X. et al. (2010). Influenza H1N1 A/Solomon Island/3/06 virus receptor binding specificity correlates with virus pathogenicity, antigenicity, and immunogenicity in ferrets. J Virol 84: 4936-4945.

Yamada S., Suzuki Y., Suzuki T. et al. (2006). Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature 444: 378-382.

Yen H., Lipatov A.S., Ilyushina N.A. et al. (2007). Inefficient Transmission of H5N1 Influenza Viruses in a Ferret Contact Model. J Virol 81: 6890-6898.

CHAPTER 6

GENERAL DISCUSSION

The influenza A viruses consist of 8 single stranded RNA gene segments associated with polymerases and nucleoproteins, a matrix as well as an envelope in which two glycoproteins (HA and NA) and an ion channel (M2) are embedded. Based on their antigenic properties, 17 different HA and 9 different NA subtypes have been identified. As 16 of the HA and all of the NA subtypes have been isolated from wild aquatic birds at some point, these animals are considered the reservoir for influenza viruses in all other species. A major concern for the public health is the sporadic transmission of avian viruses to man, possibly resulting in the emergence of a pandemic or epidemic virus. Fortunately, influenza viruses are relatively host specific and humans seem rather resistant to infection with avian influenza viruses if the virus does not undergo modifications (Beare and Webster 1991). Such adaptations can be the result from mutations in the glycoproteins or reassortment processes generating mixed human-avian influenza viruses, referred to as genetic drift and genetic shift respectively. Both processes are supposed to occur more easily in pigs. Hence, during decennia, this species has been proposed to function as either an "intermediate host" or as a "mixing vessel" (Scholtissek et al. 1985, Ito et al. 1998, Ma et al. 2009, Sriwilaijaroen et al. 2011). However, a growing number of studies have revealed that the intrinsic susceptibility of pigs to influenza viruses from entirely avian origin is low, as reflected by a low infection, replication and transmission efficiency (Shortidge et al. 1998, Choi et al. 2005, Isoda et al. 2006, Lipatov et al. 2008, De Vleeschauwer et al. 2009a; 2009b). Therefore, this thesis aimed to focus on the interactions between host factors and viral features that could determine a particular host specificity with a focus on the porcine host.

The set-up of an *ex vivo* model of the porcine respiratory tract to study influenza virus-host interactions

In order to study virus-respiratory epithelium interactions in more detail, we firstly optimized an *ex vivo* model that represented both the upper and lower porcine respiratory tract. A porcine nasal respiratory mucosa explants system derived from the pig nasal septum and the conchae was described earlier by Glorieux et al. (2007). Since the three-dimensional structure of the tracheal epithelium is very similar to that of the nasal mucosa, we cultured tracheal explants in a similar way, meaning at an air-liquid interface with culture medium at the bottom side and air on the top side (Whitcutt et al. 1988, Johnson et al. 1993). This was obtained by transferring the tissue onto an elevated metal grid. As such the morphology, viability and a situation closely resembling the *in vivo* circumstances was maintained. Our third type of tissue cultures were dissected from the tertiary bronchi. Since these bronchi are small rings it was impossible to culture them in the same way as the nasal and tracheal mucosa. Instead, we used a technique described earlier for chicken trachea organ cultures (Cook et al. 1976). Hereby the rings were maintained in cell culture tubes on a rotor device

resulting in subsequent exposure of the tissues to culture medium and air, in other words partially mimicking the air-liquid interface. Our last organ of interest, namely the lung, was the most challenging one. Because of its spongy structure it is difficult to cut through. In addition, the absence of an apical and basal orientation of lung slices makes it impossible to culture them following an air-liquid interface principle. To circumvent some particular problems, we used a technique described earlier in mice, rats and guinea pigs for the preparation of precision-cut long sections (Martin et al. 1993, Held et al. 1999, Ressmeyer et al. 2006). By filling the lung with a low melting-point agarose solution and leaving it to solidify at 4°C, we were capable of cutting thin slices manually.
Given that the explants maintain viability during several days, that they express the influenza receptor determinant variants approximating the *in vivo* situation and that they are susceptible to infection with influenza A viruses from various hosts, these systems provide some major ethical and experimental advantages. Since one pig can yield up to 30 explants of each system, the number of experimental animals can significantly be reduced. In addition, this makes it feasible to compare several viruses under identical conditions, ruling out influences of individual varying host factors such as the immune response, a different genetic background, sex, age, etc. (Bigham et al. 2011). Furthermore, virus production could be monitored over time by collecting supernatant samples at different time points.

Replication assessment of influenza viruses in the porcine respiratory tract model, as measured by virus shedding in the supernatant at 3 time points spread over 48 hours, revealed substantial differences. Notable divergences between viruses from different host origin (human, pig or birds) as well as between the avian viruses in the 4 different explant systems were detected. As might be expected from a host adapted virus, all 3 endemic European swine subtypes (H1N1, H3N2 and H1N2) screened in the model, were replicating efficiently and were reaching similar virus titers by 48 hpi. Although the absolute virus titers of the 4 explants should not be compared as such, it is clear that the replication of the swine influenza viruses is efficient in the explants derived from all 4 tissues. These observations are in accordance with *in vivo* pathogenesis experiments detecting viral replication of swine influenza viruses by virus titration, PCR, immunohistochemistry or in situ hybridization (Jung et al. 2002, De Vleeschauwer et al. 2009a, Trebbien et al. 2011). The virus replication of the human H1N1 and H3N2 subtypes was similar to that of a typical swine influenza virus, except that the titers of the human H1N1 were consistently lower than those of the H3N2. Regarding the numerous reports of serological evidence for the circulation of human influenza viruses in pig populations worldwide, it is no surprise that the human influenza viruses replicate rather efficiently in pig tissues (Harkness et al. 1972, Aymard et al. 1980, Ottis et al. 1982, Peiris et al.

2001, Rith et al. 2012). Additionally, a comparative pathogenesis study in pigs inoculated with reverse genetics generated pandemic H1N1 1918 and an early swine H1N1 isolate of 1930 (defined as classical swine influenza virus), confirmed a resembling replication pattern of both viruses in this host (Weingartl et al. 2009). Based on an experiment by Landolt et al. (2003) we need to take into consideration that some human influenza viruses might have an intrinsically higher chance of adapting to pigs than others. Evaluating the kinetics and the absolute levels of nasal virus shedding of a triple reassortant swine H3N2 and an entirely human H3N2 influenza virus (albeit isolated from a pig in Canada), they observed remarkable differences between both viruses.

The significantly lower virus titers of all avian influenza viruses in nasal, tracheal and bronchial explants, compared to the swine isolates, illustrated that these avian viruses were hampered in their replication in this porcine respiratory epithelium. These findings were in concert with all previously performed replication studies of AIVs in pigs (Shortidge et al. 1998, Choi et al. 2005, Isoda et al. 2006, Lipatov et al. 2008, De Vleeschauwer et al. 2009a; 2009b, Trebbien et al. 2011). The extremely low virus titers in nasal explants (NE) and tracheal explants (TE) even made us doubt whether the infectious viruses detected in the supernatant were the result of active viral replication or of the release of attached viruses instead. Therefore, we also inoculated fixed explants, which rendered a kinetic curve that was immediately decreasing in contrast with the slightly increasing slope observed in unfixed explants. At the level of the lung explants, the difference in virus titers between the swine and avian influenza titers became minimal, confirming the pronounced susceptibility of lung tissue for AIV earlier reported *in vivo* (Lipatov et al. 2008, De Vleeschauwer et al. 2009a; 2009b, Trebbien et al. 2011). The infected cells as detected by IHC assured that the virus titers were the result of replication in the epithelial cells and not, for example, from cells in the submucosa. As the entire explant is in contact with inoculum during the incubation stage, this could have theoretically been the case. These experiments allowed us to conclude that our *ex vivo* model provides a good alternative to study influenza virus-host interactions in more detail as well as the susceptibility of pigs to influenza viruses isolated from different hosts according to the three R's principle.

Expression of influenza virus sialic acid receptors in pigs

The low replication potential of the avian influenza viruses in nasal and tracheal explants were inconsistent with the "mixing vessel theory" and with the, until then, identified influenza receptors in the pig trachea (Ito et al. 1998, Suzuki et al. 2000). The receptor determinants for influenza viruses are terminal Sia which are usually bound to galactose in an α2-3 or α2-6 configuration. The expression of both linkage variants is known to differ between animal species and within one species between the different tissues and cell types (Ito et al. 1998, Suzuki et al. 2000, Wan and Perez 2006,

Shinya et al. 2006, Guo et al. 2007, Nicholls et al. 2007, Kuchipudi et al. 2009, Kimble et al 2010, Pillai and Lee 2010). In accordance, influenza viruses isolated from a particular host will preferentially bind the configuration variant predominantly expressed in that host. This means that bird influenza viruses prefer binding to α2-3 linkages, whereas human and swine isolates prefer α2-6 linkages. By using plant lectins SNA and MAA, that differentially bind Sia α2-6 and α2-3 linkages respectively, Ito et al. (1998) and later Suzuki et al. (2000) had shown the abundant presence of both linkages in the pig trachea. As this was not matching with our low avian influenza replication in nasal and tracheal explants, we decided to repeat the lectin histochemistry on all explant systems and freshly collected tissues in parallel. The α2-6 linked Sia were expressed abundantly on all epithelial linings, yet the α2-3 linked variant was virtually not detected on the nasal or tracheal epithelial lining and became progressively more expressed from the bronchi down to the alveoli. Although this distribution pattern was confirmed by Nelli et al. (2010) and Trebbien et al. (2011), lectin results must be interpreted with care. Variations depending on the lectin batches or the supplier could occur (Nicholls et al. 2007), different isotypes of MAA exist (Geisler and Jarvis 2011), a fraction of Sia is not covered by lectin binding and furthermore the binding of the lectins is not 100% selective (including binding to unsialylated structures). The latter has become more and more apparent as well as defined by screening the lectins in glycan microarrays (Smith et al. 2010, Song et al. 2011). An alternative method to define the glycan profile in a tissue of interest is provided by mass spectrometry (MALDI-TOF) on chromatography purified glycans. Hereby, tissue homogenates are prepared and used for the extraction of the glycans of interest. By comparing the sensitivity to digestion by linkage specific sialidases, the proportion of both linkage variants can be determined. Sialidase S is used to specifically cleave α2-3 linked Sia, while sialidase A cleaves both α2-3 and α2-6 linked Sia. Although this is a more precise method in terms of identification and quantification of glycans than lectin histochemistry, this technique has the following major drawback (Sriwilaijaroen et al. 2011, Nicholls et al. submitted for publication). As long as the epithelium cannot be dissected out separately from the surrounding tissues, it is impossible to differentiate whether the detected glycans are present in the epithelial or in the submucosal tissues.

Even though our glycan expression results are matching with literature findings and replication efficiency of viruses with a particular receptor specificity, we need to consider that the presence of a particular Sia variant does not automatically imply a functional relevance for influenza virus infection. Over the years, more and more researchers have reported the infection of cells lacking (the appropriate) Sia (Stray et al. 2000, Glaser et al. 2007, Bateman et al. 2008, Oshansky et al. 2011). In addition, double staining of lung tissues from infected pigs with lectins and NP antibodies clearly showed the absence of Sia in the infected and surrounding regions. Such observations raised even

more questions on the initiation of influenza infections and the mechanism of reassortment processes (Trebbien et al. 2011). Undoubtedly, Sia functions as a receptor determinant for influenza viruses but perhaps only function during primary attachment to the epithelium. Tighter binding and subsequent internalization may require interaction with a coreceptor or another internalizing factor. Similar entry mechanisms have been described for numerous viruses: hepatitis C virus (Cormier et al. 2004), PRRSV (Van Gorp et al. 2008), HIV-1 (Deng et al. 1996), etc. In conclusion, we demonstrated that the avian-like Sia receptor is not as widely distributed in the porcine host as believed earlier, particularly not in the upper respiratory tract.

Despite the poor expression of α2-3 Sia in the pig, it has not been pinpointed to what extent the low replication efficiency of avian influenza viruses in this host is due to the α2-3 Sia binding preference of avian influenza viruses. Instead of mismatches between viral HA and its receptors, later steps in the replication cycle could be limiting factors as well. The import of NP and polymerases into the nucleus, for example, depends on binding with host specific importins (Watanabe et al. 2010). By comparing the replication and transmission of reverse genetic generated A/Hong Kong/1/68 (R1-HK) and its avian counterpart with two substitutions in the HA (R2-HK) we tackled this question. A/Hong Kong/1/68 (H3N2) is a typical example of a human pandemic virus that emerged by reassortment of an avian and human influenza virus. The adaptation of this novel virus to the human host was accompanied by 7 mutations in the avian HA: R62I, D81N, N92K, A144G, N193S, Q226L and G228S. By an extended glycan microarray we confirmed the strong binding of R1-HK (identical to A/Hong Kong/1/68) to α2-6 linked Sia, whereas R2-HK with L226Q and S228G substitutions preferably bound α2-3 linked Sia. Earlier characterization of these viruses in cultures of human airway epithelium had already revealed a distinction in replication efficiency as well as in cell tropism. Although neither of the viruses had an all-or-none preference, R2-HK predominantly infected ciliated cells whereas R1-HK preferred non-ciliated cells (Matrosovich et al. 2007). Yet, another study by Thompson et al. (2006), using a similar cell culture system, demonstrated that human H3N2 isolated in 1969 infected proportionally more ciliated cells than human H3N2 isolates from 1999 or 2000. Before using both viruses for *in vivo* studies, we assessed them in our porcine respiratory explants systems together with two control viruses: A/swine/Flanders/1/98 (H3N2) and A/duck/Italy/3139-1/06 (H3N2). The above described replication differences between R1-HK and R2-HK were also observed in the nasal, tracheal and bronchial explants regardless the inoculation doses used. Yet, comparing the recombinant viruses with the control viruses revealed lower replication of R1-HK than of A/swine/Flanders/1/98 (H3N2) and higher replication of R2-HK than of A/duck/Italy/3139-1/06 (H3N2). A plausible explanation for these observations could be a differential recognition of

additional glycan structural features, apart from the distinction between α2-3 or α2-6 linkages (Gambaryan et al. 2004; 2005). Also other host specific viral features, apart from the receptor binding preferences, could be the cause of these observed differences. To differentiate which was the case here, also the control viruses should be submitted to the same glycan microarray as R1-HK and R2-HK. Although the virus titers of R1-HK in NE and TE were lower than of A/swine/Flanders/1/98 (H3N2), nasal shedding kinetics of inoculated pigs and of contact pigs were almost perfect copies for R1-HK and A/swine/Flanders/1/98 (H3N2) (De Vleeschauwer et al. 2009b).

Hemagglutinin properties affecting transmission of influenza viruses in pigs

Transmission of influenza viruses between animals could in theory occur by 3 different routes: (a) by direct contact with infected animals, (b) by contact with contaminated objects (fomites) or (c) by airborne transmission. For the latter mode of transmission, a differentiation is made between small particle aerosols (typically <5µm) or large respiratory droplets (typically >5µm), because only the former are believed to be directly deposited in the alveoli (Sorrell et al. 2011). Environmental factors such as temperature and humidity were also shown to influence the transmission efficiency in a guinea pig model (Lowen et al. 2007). Which of the suggested transmission routes is more important in pigs is not defined. Only recently, Mubareka et al. (2009) showed that the transmission by fomites was inefficient in their guinea pig model. Although our experimental set-up was permissive to all possible transmission modes, the R2-HK was still clearly hampered in its transmission to contact pigs as no virus was detected in any of the nasal swabs and seroconversion was detected in one pig only. Regarding the much lower nasal virus shedding of R2-HK compared to R1-HK by inoculated animals, we can assume that the contact pigs in the R2-HK group were exposed to lower viral loads. This in itself could already explain the lack of transmission although other options should be considered. Studying the transmission of a similar 226-228 mutant in ferrets, Roberts et al. (2011) attributed the lack of transmission of this virus to a lower infectivity *in vivo* rather than to decreased viral shedding. Another remarkable observation during our transmission experiment, was the drop in nasal shedding of R2-HK at 2 and 3 dpi, which later on recovered. By performing virus titrations of different organs during 5 subsequent days, we were hoping to find a distinction in virus production around this period. However, the virus titers of R2-HK were lower than of R1-HK in all tissues, except for the olfactory part of the nasal mucosa, during the entire period monitored. Regarding the time point of this virus drop, a difference in the induced innate immunity reactivity seems a plausible explanation. *In vitro* studies using HPAIV H5N1 wild type and a Q226L, G228S mutant revealed that the virus with the avian-like binding specificity induced higher levels of cytokines and chemokines in human

dendritic cells, macrophages and human tracheobronchial epithelial cells (Ramos et al. 2011). The relevance of such differences *in vivo* are yet to be elucidated.

Considering the numerous reports of differences in the cell types that are infected by viruses with different receptor binding properties (Matrosovich et al. 2004; 2007, Thompson et al. 2006, Wan and Perez 2007), we focused in more detail on whether the low replication of R2-HK in most pig tissues *in vivo* could result from infection of other target cells. Yet again, the difference observed between R1-HK and R2-HK, was the number of cell infected with the latter and not the type of cells infected by both viruses. Some notable differences between the two viruses were observed in the interactions with the superficial mucus layer and SP-D. During the infection reduction test performed in our lab, we observed a stronger decline of the R1-HK infectivity than that of R2-HK. Following the hypothesis that pig mucus contains alot of α2-3 linked terminal Sia, these results are contradicting. The composition of mucus, produced by goblet cells and submucosal glands, so far is not well defined in pigs. The mucus glycoproteins, which contain mainly O-linked glycans, are associated with various macromolecules. Based on the lectin staining results found in literature and in our lab, SNA binding mostly corresponded with the location of goblet cells and submucosal glands (Nelli et al. 2010, Van Poucke 2010, Punyadarsaniya 2011, Trebbien et al. 2011). This indicates that at least a fraction of the pig mucins is rich in α2-6 linked terminal Sia. This could explain why the R1-HK viruses seemed to interact stronger with the mucus than R2-HK. In addition, a balance with the neuraminidase activity may be of crucial synergism for the virus to be subsequently released again and to penetrate deeper through the layer (Matrosovich et aL. 2004). Based on these results and on viral replication results *in vivo*, we hypothesize that interactions with sialylglycoconjugates may help host adapted influenza viruses to pass through the Sia rich mucus layer that covers the viral target cells in the epithelium. A strategy to tackle this question would include of a more detailed identification of the different pig mucus components. Since the major gel-forming units of mucus are identified as mucins, being large glycoproteins abundantly decorated with carbohydrate groups such as galactose, fucose, N-acetylgalactosamine and Sia, we would start with separating differenct components on a Western blot. A similar technique was used before by Ehre et al. (2012) to characterize overexpression of mucin 5Ac in mice. In a first step, antibodies such as those recognizing MUC5AC (which has been shown to be present in pigs), MUC1, MUC2, MUC4, MUC5B etc. are needed to identify the different bands. Subsequently, labeled SNA and MAA can be used to provide a hint of the most abundant Sia variant and to colocalize this with the earlier identified bands. Finally, purified, labeled and standardized amounts of HA from R2-HK and R1-HK can be used for staining as well. These could be obtained by transfection of cells with plasmids containing the R1-HK and R2-HK HA. If our hypothesis

is correct, we should observe a strong binding of SNA to at least one band, which on his turn should correspond with the site where the R1-HK HA attaches best.

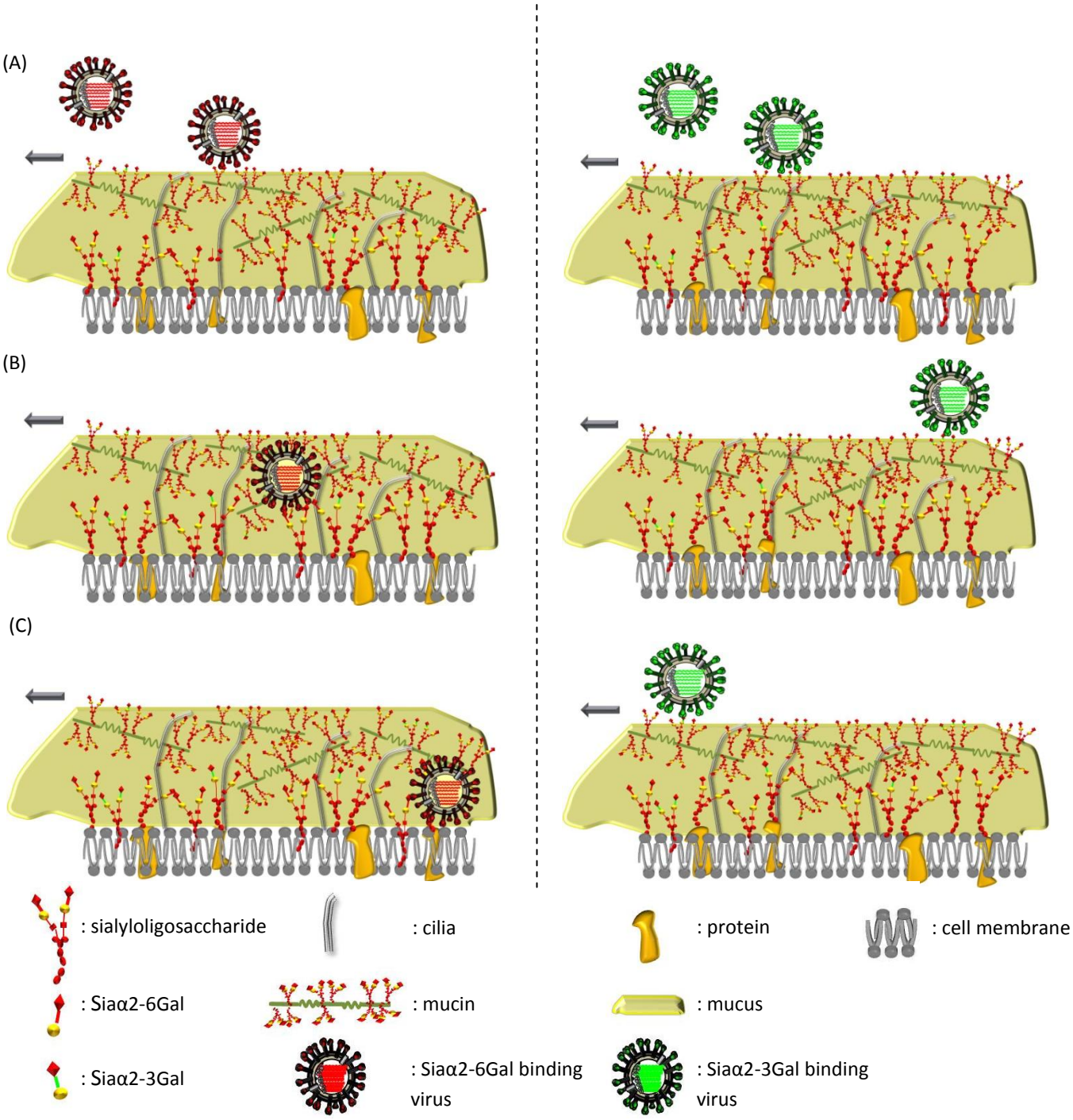

Figure 1: Schematic proposal of the different stages of virus interaction of influenza viruses, distinct in receptor binding specificity, with host components present in the upper respiratory tract. In all panels the direction of mucociliairy transport, resulting from the orchestrated beating of the numerous cilia, is indicated by the dark arrow. In panel (A) the entrance of the viruses with human- (left column) or avian-like (right column) receptor binding specificity occurs similar. The virions, introduced in the nasal cavity or tracheal lumen will gradually lose velocity by interacting with terminal sialic acids (Sia) present on the surface of the mucus layer. This binding might be stronger for human-like than for avian-like viruses, resulting in a faster and tighter attachment of the former. In a next step (B) the human-like virus uses the Sia interactions to make its way through the mucus layer, most likely in combination with a well balanced catalytic NA activity that prevents the captation of the particle. The avian-like virus on the other hand, although attached to the mucus surface has a too low receptor avidity to move deeper down the mucus layer. The numerous α2-6 linked terminal Sia on glycoproteins or glycolipids of the target cell, as shown in (C) provide a bridge to pool the human-like virus closer in the vicinity of the cell membrane, ultimately leading to internalization of the virion. By now the avian-like virus is, attached to decoy receptors, removed from the respiratory tract as a result of the mucociliairy clearance.

In chapter 5 we focused on the possible contribution of the 5 remaining amino acid substitutions in the HA of A/Hong Kong/1/68 to the adaptation to a mammalian host by assessing R5-HK in our standard transmission model. Although the nasal excretion profile of the R5-HK inoculated pigs did not significantly differ from R1-HK, transmission did. The first contact pigs shedding virus in nasal swabs did not appear until 6 days post contact. In addition, there was one contact pig that never excreted virus although seroconversion was demonstrated in that same animal. One particular mutation that was isolated by repetition in 3 out 5 contact pigs, namely D60G, provided an indication for a possible functional process underlying inefficient transmission of R5-HK. Because amino acid 60 is located within the vestigial esterase subdomain of HA1 we propose a possible contribution to the spring-loaded mechanism of HA (Figure 2). After attachment and internalization of the virus, it is the low pH inside the endosome (pH 5–6), that triggers the fusion reaction between the viral envelope and the endosomal membrane. At low pH, two subsequent major conformational changes in the HA spike are induced. Initially the electrostatic interaction between the vestigial esterase domain of HA1 and the B-loop of HA2 needs to be interrupted. This occurs by protonation of positively charged amino acids in the vestigial esterase subdomain (Rachakonda et al. 2007, Xu and Wilson 2011). Only then the loop to helix transition of the B-loop can take place, resulting in the protrusion of the fusion peptide of HA2 which is previously buried within the stem of the HA trimer (Chen et al. 1999, Smith et al. 2004). This is a key step in the viral infection mechanism which is characterized by an optimal pH. Optimal, meaning that this pH should not be too high so that the virus is not undergoing the conformation changes outside the target cell and at the same time not too low so that fusion occurs before the virus is destroyed in the endosome. Most likely this optimal pH is also host dependent, in relation with the species physiology and the pathogenesis of influenza in a particular host. Comparison of the remaining infectivity after NH_4Cl treatment of all reverse genetic generated viruses (R1-HK, R2-HK, R5-HK and R7-HK) and the naturally isolated mutant R5+1-HK showed clear differences. R1-HK and R7-HK maintained similar levels of infectivity, which could depict an optimal balance in a mammalian and avian host respectively. We clearly observed that the change in receptor specificity of R2-HK resulted in much lower infectivity at increased pH compared to R1-HK. This is a puzzling observation as both 226 and 228 are strictly located in the RBS. Yet, this finding reinforces the thought that at least one of the other 5 amino acid mutations were introduced to compensate this change. Since the R5+1-HK infectivity was significantly different from R5 we, furthermore, confirmed that the D60G mutation is functionally affecting pH sensitivity. Although these results suggest that efficient transmission of influenza viruses in pigs requires, besides a proper receptor binding property also an optimal pH of HA activation, this concept needs further research to be proven and unraveled. Further experiments include a repetition of the pig transmission experiment

under identical circumstances with R5+1-HK to confirm a more efficient transmission of this virus. Also full sequencing of the R5+1-HK genes would be required to exclude that additional adaptational mutations have occurred. If this is affirmative, introduction of the D60G mutation into R5-HK should result in a virus with identical phenotype as R5+1-HK. Finally, more assays that really measure membrane fusion under well defined pH conditions, similar to the ones by Su et al. (2009), are needed. In their cell-cell fusion assay they measured the fusion between Hela cells cotransfected with a plasmid expressing HA and Tat proteins and MDCK cells harboring a Tat-inducible β-galactosidase reporter system under different pH conditions. When fusion of both cell types occurred, the galactosidase activity was colorimetricly analyzed.

The results from the transmission experiments in pigs provided evidence that although the transmissibility of an influenza virus is defined in part by the binding specificity of its HA, other properties of the HA can contribute as well.

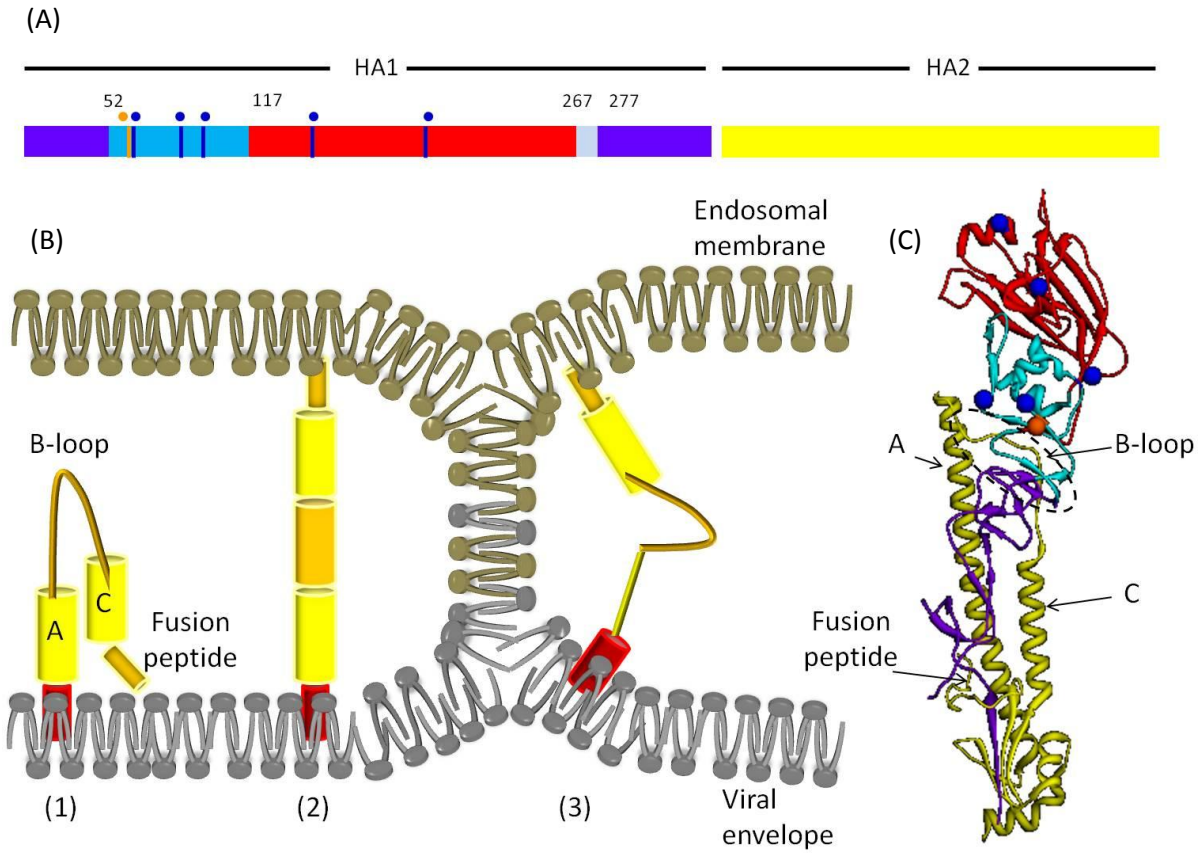

Figure 2: (A) Schematic representation of the HA protein after proteolytic cleavage into HA1 and HA2 subunits. The two subunits remain linked by a disulphide-bridge. Within HA1, the receptor binding sub-domain is in red, the vestigial esterase sub-domain is in blue and the F' fusion sub-domain is in purple (After Ha et al. 2002). The numbers of the amino acids that make up the beginning of each sub-domain are indicated. The locations of the 5 amino acids that differ between R1-HK and R5-HK are highlighted in blue: I62R, N81D, K92N, G144A and S193N. The additional D60G mutation isolated from contact pigs in the vestigial esterase sub-domain is highlighted in orange. (B) Schematic representation of the different stages of the spring-loaded mechanism resulting in the protrusion of the HA2 fusion peptide. One (1) shows the native confirmation of the HA2 monomer with the N-terminal fusion peptide buried in the core of the structure. Decreasing pH conditions in (2) trigger a loop to helix transformation of the B-loop (amino acids 55-76), resulting in the incorporation of the fusion peptide in the endosomal membrane. In (3), a helical to loop transformation of (A), composed of amino acids 106-112, pulls the fusion peptide and locally bends the membranes towards eachother. This will ultimately result in the disruption of the bilipid-layer and the release of viral RNP's into the cytoplasm. (C) shows the ribon structure of one protomer of HA, with sub-domains colored as in panel A (the image was created with Chimera version 1.5.3). Considering the location of the D60G mutation, this change could influence the pH and mechanism of fusion activation because the vestigial esterase sub-domain interacts with the B-loop of the HA2 stalk-domain. As long as the electrostatic interaction between the vestigial esterase sub-domain and the B-loop is maintained, the transition from loop to helix of the latter will not occur.

Design of antiviral agents directed against the hemagglutinin and neuraminidase

A sound comprehension of the virus-host interactions at the molecular level is not only important for the prediction of viral changes that might lead to interspecies transmission but also for the design of mechanism-based antiviral drugs. Such drugs can be applied as a first aird to control newly emerging influenza viruses while the production of a new vaccine is still in progress. Therefore, the drug requires activity against all influenza viruses, regardless the host of origin or the subtype to which it

belongs. Two major groups of drugs can be distinguished, those directly interfering with viral components and those acting on cellular host mechanism that are used by the virus for viral replication. Drugs classified under the first group, will be mainly directed against the HA surface glycoprotein, the M2-ion channel or the NA, targeting the attachment, the fusion or the release of virus particles respectively. Currently only drugs belonging to the latter to types are available on the market. The earliest compounds used for therapeutic and prophylactic treatment, such as amantadine and rimantadine, are sterically blocking the interior of the M2-ion channel. As a consequence, influx of protons is prevented and the fusion of the viral envelope and the endosomal membrane is stopped. The emergence of widespread resistance to these drugs, mostly associated with a S31N mutation, together with significant adverse affects have restricted their use (Bright et al. 2006, De Clercq 2006, Deyde et al. 2007). The second generation anti-virals belong to the group of the neuraminidase inhibitors, with zanamivir and oseltamivir as the best known examples. These compounds are transition state analogous of the Sia that form complexes with the active site of the enzyme in a non-covalent way (Kim et al. 1997). Since the amino acids which line the walls of this binding site are highly conserved, sporadic escapate mutations that arise in response to the use of these agents, are regularly accompanied by a compromised viral growth and limited transmissibility. The design of a new class of NA inhibitors based on covalent binding of the Sia intermediate, is recently being explored (Kim et al. 2013).

Another potential site of intervention, and considering its diversity also the most challenging one, is the HA. This type of agents need to target highly conserved regions of the HA for two reasons: to have a drug active against multiple subtypes of viruses and to prevent the emergence of resistant strains. Considering the very specific and multivalent based interaction of HA with the sialyloligosaccharides, an effective drug will need to bind HA in competition with endogenous Sia. Apart from the type of Sia linkage with Gal, also the length of the glycan and the number of terminal Sia present, may be a determining factor for the strength of binding. Although some new compounds linked to a liposome carrier can bind in a multivalent and more flexible way to HA (Hendricks et al. 2013), I believe that there is still a long way to go before this type of drugs will meet all the requirements (if they ever will). At last, the newest antiviralcompounds under development either inhibit the function of the PA (DuBois et al. 2012) or bind to the nuclear localization signal of the NP protein (Aida et al. 2012).

References

Aida Y., Sasaki Y., Hagiwara K. (2012). Discovery of Novel Antiviral Agents Directed Against the Influenza A Virus Nucleoprotein. In: Antiviral Drugs – Aspects of Clinical Use and Recent Advances. Arbuthnot P. (Ed). ISBN: 978-953-51-0256-4.

Aymard M., Brigaud M., Fontaine M. et al. (1980). Relations between swine influenza and human influenza: data obtained from serological studies in France. Annales de Virologie 13: 527-528.

Bateman A.C., Busch M.G., Karasin D.A. et al. (2008). Amino Acid 226 in the Hemagglutinin of H4N6 Influenza Virus Determines Binding Affinity for α2,6-Linked Sialic Acid and Infectivity Levels in Primary Swine and Human Respiratory Epithelial Cells. J Virol 82: 8204-8209.

Beare A.S. and Webster R.G. (1991). Replication of avian influenza viruses in humans. Arch Virol 119: 37-42.

Bigham A. W., Buckingham K.J., Husain S. (2011). Host Genetic Risk Factors for West Nile Virus Infection and Disease Progression. PLoS ONE 6: doi:10.1371/journal.pone.0024745.

Bright R.A., ShayD.K., Shu B. et al. (2006). Adamantane resistance among influenza A viruses isolated early during the 2006-2006 influenza season in the United States. JAMA 295: 891-894.

Choi Y.K., Nguyen T.D., Ozaki H. et al. (2005). Studies of H5N1 influenza virsu infecton of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol 79: 10821-10825.

Chen J., Skehel J.J., Wiley D.C. (1999). N- and C-terminal residues combine in the fusion-pH influenza hemagglutinin HA2 subunit to form an N cap that terminates the triple-stranded coiled coil. PNAS 96: 8967-8972.

Cook J.K.A., Darbyshire J.H., Peters R.W. (1976). The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. Arch Virol 50: 109-118.

Cormier E.G., Tsamis F., Kajumo F. et al. (2004). CD81 is an entry coreceptor for hepatitis C virus. PNAS 101: 7270-7274.

De Clercq E. (2006). Antiviral agents active against influenza A viruses. Nature 5: 1015-1025.

Deng H., Lui R., Ellmeier W. et al. (1996). Identification of a major co-receptor for primary isolates of HIV-1. Nature 381: 661-666.

Deyde V.M., Xu X., Bright R.A. et al. (2007). Surveillance of Resistance to Adamantanes among Inluenza A(H3N2) and A(H1N1) Viruses Isolated Worldwide. J Inf Dis 196: 249-257.

De Vleeschauwer A., Atanasova K., Van Borm S. et al. (2009a). Comparative pathogenesis of an avian H5N2 and a swine H1N1 influenza virus in pigs. Plos ONE 4: doi:10.1371/journal.pone.0006662.

De Vleeschauwer A., Van Poucke S., Braeckmans D. et al. (2009 b). Efficient transmission of swine-adapted but not wholly avian influenza viruses between pigs and from pigs to ferrets. J Inf Dis 200: 1184–1192.

DuBois R.M., Slavish P.J., Baughman B.M. et al. (2012). Structural and Biochemical Basis for Development of Influenza Virus Inhibitors Targeting the PA Endonuclease. PLoS Pathog 8: doi:e1002830. doi:10.1371/journal.ppat.1002830.

Ehre C., Worthington E.N., Liesman R.M. et al. (2012). Overexpressing mouse model demonstrates the protective role of Muc5ac in the lungs. PNAS www.pnas.org/cgi/doi/10.1073/pnas.1206552109

Gambaryan A.S., Tuzikov A.B., Pazynina G.V. et al. (2004). H5N1 chicken influenza viruses display a high binding affinity for Neu5Acalpha2-3Galbeta1-4(6-HSO3)GlcNAc-containing receptors. Virol 326: 310-316.

Gambaryan A., Yamnikova S, Lvov D. et al. (2005). Receptor specificity of influenza viruses from birds and mammals: new data on involvement of the inner fragments of the carbohydrate chain. Virol 334: 276-283.

Geisler C. and Jarvis D.L. (2011). Effective glycoanalysis with MAackia amurensis lectins requires a clear understanding of their binding specificities. Glycobiology 21: 988-993.

Glaser L., Conenello G., Paulson J. et al. (2007). Effective replicatin of human influenza viruses in mice lacking a major α2,6 sialyltransferase. Virus Res 126: 9-18.

Glorieux S., Van den Broeck W., van der Meulen K.M. et al. (2007). In vitro culture of porcine respiratory nasal mucosa explants for studying the interaction of porcine viruses with the respiratory tract. J Virol Methods 142: 105-112.

Guo C., Takahashi N., Yagi H. et al. (2007). The quail and chicken intestine have sialyl-galactose sugar chains responsible for the binding of influenza A viruses to human type receptors. Glycobiology 17: 713-724.

Ha Y., Stevens D.J., Skehel J.J. et al. (2002). H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. EMBO J 21: 865-875.

Harkness J.W., Schild G.C., Lamont P.H. et al. (1972). Studies on relationships between human and porcine influenza 1. Serological evidence of infection in swine in Great Britain with an influenza A virus antigenically like human Hong Kong/68 virus. Bull W H O 46: 709-719.

Held H.D., Martin C., Uhlig S. et al. (1999). Characterization of airway and vascular responses in murine lungs. Br J Pharmacol 126: 1191-1199.

Hendricks G.L., Weirich K.L., Viswanathan K. et al. (2013). Sialylneolacto-N-tetraose c (LSTc)-bearing Liposomal Decoys Capture Influenza A Virus. J Biol Chem 288: 8061-8073.

Isoda N., Sakoda Y., Kishida N. et al. (2006). Pathogenicity of a highly pathogenic avain influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. Arch Virol 151: 1267-12679.

Ito T., Nelson J., Couceiro S.S. et al. (1998). Molecular basis for the generation in pigs of influeza A viruses with pandemic potential. J Virol 72: 7367-7373.

Johnson L.G., Dickman K.G., Moore K.L. et al. (1993). Enhanced Na^+ transport in air-liquid interface culture system. Am J Physiol 264: L560-L565.

Jung T., Choi C., Chae C. (2002). Localization of Swine Influenza Virus in Naturally Infected Pigs. Vet Pathol 39: 10-16.

Jung K, Ha Y., Chae C. (2005). Pathogenesis of Swine Influenza Virus Subtype H1N2 Infection in Pigs. J Comp Path 132: 179-184.

Kimble B., Nieto G.R., Perez D.R. (2010). Characterization of influenza virus sialic acid receptors in minor poultry species. Virol J 7: doi:10.1186/1743-422X-7-365.

Kim C.U., Lew W., Williams M.A. et al. (1997). Influenza Neuraminidase Inhibitors Possessing a Novel Hydrophobic Interaction in the Enzyme Active Site: Design, Synthesis, and Structural Analysis of Carbocyclic Sialic Acid Analogues with Potent Anti-Influenza Activity. J Am Chem Soc 119: 681-690.

Kim J., Resende R., Wennekes T. et al. (2013). Mechanism-Based Covalent Neuraminidase Inhibitors with Broad-Spectrum Influenza Antiviral Activity. Science 340: doi: 10.1126/science.1232552.

Kuchipudi S.V., Nelli R., Whie G.A. et al. (2009). Differences in influenza virus receptors in chickens and ducks: Implications for interspecies transmission. J Mol Genet Med 3: 143-151.

Landolt G.A., Karasin A.I., Phillips L. et al. (2003). Comparison of the Pathogenesis of Two Genetically Different H3N2 Influenza A Viruses in Pigs. J Clin Microbiol 41: 1936-1941.

Lipatov A.S., Kwon Y.K., Sarmento L.V. et al. (2008). Domestic pigs have low susceptibility to H5N1 highly pathogenic avian influenza viruses. PLoS Pathog 4: doi: 10.1371/journal.ppat.1000102.

Lowen A.C., Mubarkea S., Steel J. et al. (2007). Influenza Virus Tansmission Is Dependent on Relative Humidity and Temperature. PLoS Pathog 3: doi:10.1371/journal.ppat.0030151.

Martin C., Uhlig S., Ullrich V. et al. (1996). Videomicroscopy of metacholine induced contraction of individual airways in precision-cut lung slices. Eur Resp J 9: 2479-2487.

Matrosovich M.N., Matrosovich T.Y., Gray T. et al. (2004a). Human and avian influenza viruses target differenct cell types in cultures of human airway epithelium. PNAS 101: 4620-4624.

Matrosovich M.N., Matrosovich T.Y., Gray T. (2004b). Neuraminidase Is Important for the Initiation of Influenza Virus Infection in Human Airway Epithelium. J Virol 78: 12665-12667.

Matrosovich M., Matrosovich T., Uhlendorff J. et al. (2007). Avian-virus-like receptor specificity of the hemagglunin impedes influenza virus replication in cultures of human airway epithelium. Virol 361: 384-390.

Ma W., Kahn R.E., Richt J.A. (2009). The pig as a mixing vessel for influenza viruses: Human and veterinary implications. J Mol Genet Med 1: 158-166.

Mubareka S., Lowen A.C., Steel J. et al. (2009). Transmission of Influenza Virus via Aerosols and Fomits in the Guinea Pig Model. J Inf Dis 199: 858-865.

Nelli R.K., Kuchipudi S.V., White G.A. et al. (2010). Comparative distribution of human and avian type sialic acid influenza receptors in the pig. Vet Res 6: doi:10.1186/1746-6148-6-4.

Nicholls J.M., Bourne A.J., Chen H. et al. (2007). Sialic acid receptor detection in the human respiratory tract: evidence for widespread distribution of potential binding sites for human and avian influenza viruses. Resp Res 8: doi:10.1186/1465-9921-8-73.

Oshansky C.M., Pickens J.A., Bradley K.C. et al. (2011). Avian Inlfuenza Viruses Infect Primary Human Bronchial Epithelial Cells Unconstrained by Sialic Acid α2,3 Residues. PLoS ONE 6: doi: 10.1371/journal.pone.0021183.

Ottis K., Bachmann P.A., Webster R.G., et al. (1982). Human influenza A viruses in pigs: isolation of a H3N2 strain antigenically related to A/England/42/72 and evidence for continuous circulation of human viruses in the pig population. Arch Virol 73: 103-108.

Peiris J.S., Guan Y., Markwell D. et al. (2001). Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern China: potential for genetic reassortment? J Virol 75: 9679-9686.

Pillai S.P.S. and Lee C.W. (2010). Species and age related differences in the type and distribution of influenza virus receptors in different tissues of chickens, ducks and turkeys. Virol J 7: doi:10.1186/1743-422X-7-5.

Punyadarsaniya D., Liang C., Winter. et al. (2011). Infection of Differentiated Porcine Airway Epithelial Cells by Influenza Virus: Differential Susceptiblity to Infection by Porcine and Avian Viruses. PloS ONE 6: doi:10.1371/journal.pone.0028429.

Rachakonda S.P., Veit M., Korte T. et al. (2007). The relevance of salt bridges for the stability of the influenza virus hemagglutinin. FASEB J 21: 995-1002.

Ramos I., Bernal-Rubio D., Dunham N. et al. (2011). Effects of Receptor Binding Specificity of Avian Influenza Virus on the Human Innate Immune Response. J Virol 85: 4421-4431.

Ressmeyer A.R., Larsson A.K., Vollmer E. et al. (2006). Characterization of guinea pig precision cut lung slices: comparison with huma tissues. Eur Resp J 28: 603-611.

Rith S., Netrabukkana P., Sorn S. et al. (2012). Serological evidence of human influenza virus infections in swine populations, Cambodia. Infl Other Resp Vir: doi: 10.1111/j.1750-2659.2012.00382.

Roberts K.L., Shelton H., Scull M. et al. (2011). Lack of transmission of a human influenza virus with avian receptor specificity between ferrets is not due to decreased virus shedding, but rather a lower infectivity in vivo. J Gen Virol 92: 1822-1831.

Scholtissek C., Bürger H., Kistner O. et al. (1985). The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virol 147: 287-294.

Shinya K., Ebina M., Shinya Y. et al. (2006). Influenza virus receptors in the human airway. Nature 440: 23.

Shortridge K.F., Zhou N.N., Guan P. et al. (1998). Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virol 252: 331-342.

Smith A.E. and Helenius A. (2004). How viruses enter animal cells. Science 304: 237-242.

Smith D.F., Song X., Cummings R.D. (2010). Use of Glycan Microarrays to Explore Specificity of Glycan-Binding Proteins. Methods in Enzymol 480: 417-444.

Song X., Yu H., Chen X. et al. (2011). A Sialylated Glycan Microarray Reveals Novel Interactons of Modified Sialic Acids with Proteins and Viruses. J Biol Chem 286: 31610-31622.

Sorrell E.M., Schrauwen E.J.A., Linster M. (2011). Predicting 'airborne' influenza viruses: (trans-) mission impossible? Current Opinion in Virology 1: 635-642.

Sriwilaijaroen N., Kondo S., Yagi H. et al. (2011). N-Glycans from Porcine Trachea and Lung: Predominant NeuAcα2-6Gal Could Be a Selective Pressure for Influenza Variants in Favor of Human-Type Receptor. PLoS ONE 6: doi:10.1371/journal.pone.0016302.

Stray S.J., Cummings R.D., Air G.M. (2000). Influenza virus infection of desialylated cells. Glycobiology 10, 649-658.

Su B., Wurtzer S., Rameix-Welti M. et al. (2009). Enhancement of the Influenza A Hemagglutinin (HA)-Mediated Cell-Cell Fusion and Virus Entry by the Viral Neuraminidase (NA). PLoS ONE 4: doi: 10.1371/journal.pone.0008495.

Suzuki Y., Ito T., Suzuki T. et al. (2000). Sialic Acid Species as a Determinant of the Host Range of Influenza A viruses. J Virol 74: 11825-11831

Thompson C.I., Barclay W.S., Zambon M.C. et al. (2006). Infection of Human Airway Epithelium by Human and Avian Strains of Influenza A Virus. J Virol 80: 8060-8068.

Trebbien R., Larsen L.E., Viuff B.M. (2011). Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virol J doi:10.1186/1743-422X-8-434

Van Gorp H., Van Breedam W., Delputte P.L. et al. (2008). Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. J Gen Virol 89: 2943-2953.

Wan H. and Perez D.R. (2006). Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. Virol 346: 278-286.

Watanabe T., Watanabe S., Kawaoka Y. (2010). Cellular Networks Involved in the Influenza Virus Life Cylce. Cell Host & Microbe 7: 427-439.

Weingartl H.M., Albrecht R.A., Lager K.M. (2009). Experimental Infection of Pigs with Human 1918 Pandemic Influenza Virus. J Virol 83: 4287-4296.

Whitcutt M.J., Adler K.B., Wu.R. (1988). A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. In Vitro Cell Dev Biol 24: 420-428.

Xu R. and Wilson I.A. (2011). Structural Characterization of an Early Fusion Intermediate of Influenza Virus Hemagglutinin. J Virol 85: 5172-5182.

SUMMARY-SAMENVATTING

Summary

Influenza A viruses which belong to the family of the *Orthomyxoviridae*, infect a wide range of natural hosts including humans, pigs, wild birds, poultry, ferrets, etc. Because of the segmented nature of their RNA genome these viruses are characterized by frequent mutations or reassortment events (mixing of gene segments derived from 2 different influenza viruses). Both adaptational processes are brought in correlation with transmission of influenza viruses to a novel host species, not rarely from an avian to a mammalian host. Pigs, being susceptible to avian, human and swine influenza viruses are attributed an important rol in this influenza ecology either as an intermediate host or as a mixing vessel. Today, almost one century after the first well documented human H1N1 1918 pandemic, the understanding of host and viral properties making up the species barrier is still insufficient to identify new potential pandemic viruses during surveillance programmes. Furthermore, the true role of the pig in interspecies transmission remains undefined. The aims of this thesis were to optimize a representative porcine *ex vivo* respiratory tract model and to study in more detail virus-host interactions in the pig, with a focus on transmissibility.

In **Chapter 1** we started out with a general introduction on the influenza A virus, including virus structure, replication cycle, influenza ecology and pathogenesis in different host species. The second part focused in more detail on the different host and viral interacting components that are identified in literature. We concluded this chapter with an overview of pro- and contra-arguments on the special role of pigs in (human) influenza ecology.

In **Chapter 2** the fundamental reasons leading to this research were identified as well as the specific aims of this thesis. In the three subsequent chapters all research results, obtained in the context of this PhD, were reported.

Chapter 3 described the set up of an *ex vivo* model of the respiratory tract, consisting of nasal mucosa (respiratory part), trachea, bronchi and lung tissue. Besides monitoring the viability of the different systems over time also their susceptibility to different influenza isolates was assessed. While avian influenza viruses were clearly hampered in their replication in nasal and tracheal explants, this was not true for pig and human isolates. The lower down the respiratory tract, as represented by bronchial and lung explants, the smaller the replication differences observed between all viruses. The relevance of the virus titers determined in the supernatant were furthermore confirmed by immunohistochemical staining of the explants, assuring the epithelial location of infected cells. Surprised by the low replication of avian influenza viruses, we determined the expression of sialic acid receptors in the established model by lectin histochemistry, using freshly

derived tissues in parallel as a control. This revealed a limited expression of the avian-like receptor on the epithelial lining of nose, trachea and bronchus. At the lung level, this receptor was more abundantly present. The human-like receptor was profusely present along the entire respiratory tract. Finally, we repeated the lectin histochemistry following earlier described methods to exclude that our unexpected results were caused by technical differences.

In **chapter 4**, using two recombinant viruses that differed in receptor binding preference only due to two changes in the HA, we focused on the extent that the avian-like receptor binding preference of an influenza virus restricts replication in pigs. The reverse genetic generated viruses were A/Hong Kong/1/68 (human-like) and the L226Q and S228G (avian-like) variant. In a first study (chapter 4.1.) we compared the transmissibility of both viruses between pigs by introducing 6 contact pigs in a group of 6 inoculated pigs at 2 days after inoculation. Nasal virus shedding and serological responses were monitored in all pigs. All human-like infected pigs excreted virus efficiently resulting in a successful transmission to all contact pigs, as soon as one day after contact. The pigs inoculated with the avian-like virus showed lots of individual variation between pigs and an overall lower nasal shedding than the human-like group. Virus was not detected in the nasal swabs of any of the contact pigs. The inefficient transmission of the avian-like virus was accompanied by a low virus replication in all but one of the tissues of the respiratory tract, including the respiratory part of the nasal mucosa, the trachea, the bronchus and different lung lobes. In chapter 4.2. we focused in more detail on the possible mechanisms underlying the low replication of the avian-like influenza virus *in vivo* and *in vitro.* Using immunohistochemistry to localize virus infected cells in respiratory tract tissues at 2 dpi we aimed to identify whether the difference in receptor binding phenotype was leading to a diverse cell tropism. As this did not seem to be the case, we studied interactions with other host factors possibly interfering with infection outcome, such as mucus and surfactant proteins. Also a possible disbalance between the HA and NA functionality was assessed. There were no indications that the latter was true, but a divergent interaction with mucus and SP-D was noted.

In **chapter 5** we studied the transmissibility of another A/Hong Kong/1/68 variant, carrying 5 distinct mutations in the HA that correspond with a more avian-like sequence. The opposite mutations accompanied the transfer of the avian HA to the human host but were never studied in terms of functional relevance and adaptational value. The transmission results, reflecting an inefficient and delayed pattern, revealed that at least one or several of the mutations must have been contributing to the adaptation from an avian to a mammalian host. Sequencing of the HA1 isolated from contact pigs identified one additional mutation in 3 out 5 virus secreting contact pigs: D60G. Limited characterization of this isolate in an *in vitro* test with NH_4Cl suggested an influence on the pH fusion

activation of this virus, which might be another determining factor for virus transmission and host adaptation.

Chapter 6 provided a critical discussion on the results obtained in this thesis as well as possible future plans to elucidate some of the questions raised by the current research. First, the set-up of the *ex vivo* system and the correlation with virus replication and sialic acid receptors expression was discussed. Specific attention was payed to several pitfalls of the use of lectin histochemistry and potential alternative methods. Next, the need for a mammalian virus receptor specificity for efficient replication and transmission was argued as well as a hypothesis involving a differentiating role of mucus and surfactant proteins. To conclude, the mechanism of an additional biological feature conferring host adaptation was suggested.

Samenvatting

Influenza A virussen, behorende tot de familie van de *Orthomyxoviridae*, infecteren een brede waaier aan natuurlijke gastheren waaronder mens, varkens, wilde vogels, pluimvee, fretten, enz. Door het gesegmenteerde karakter van hun RNA genoom, worden ze gekenmerkt door frequente mutaties en reassorteringen (uitwisseling van gensegmenten afkomstig van 2 verschillende virussen). Beide adaptieve processen worden in verband gebracht met de transmissie van influenza virussen naar een nieuwe gastheerspecies, niet zelden van vogels naar zoogdieren. Aan varkens, gevoelig voor zowel aviaire, humane als porciene influenzavirussen, wordt hierbij een speciale rol toegeschreven als intermediaire gastheer of als mengvat. Tot op de dag van vandaag, bijna één eeuw na de eerste goed gedocumenteerde uitbraak van de humane H1N1 pandemie in 1918, is de kennis over gastheer- en viruseigenschappen die bepalend zijn voor gastheerspecificiteit nog te beperkt om potentiële pandemische virussen te identificeren tijdens surveillance studies. Ook de exacte rol van het varken in de interspecies transmissie is nog onopgehelderd. De algemene doelstellingen van deze thesis waren de optimalisatie van een representatief respiratoir *ex vivo* model van het varken evenals het verkrijgen van meer inzichten betreffende influenza virus-gastheer interacties bij het varken, in het bijzonder deze bepalend voor transmissie.

In **hoofdstuk 1** gingen we van start met een algemene inleiding over het virus, waarbij o.a. structuur, replicatiecyclus, influenza ecologie en pathogenese bij verschillende diersoorten werden besproken. In het tweede deel richtten we ons op de virale componenten en gastheerfactoren bepalend voor gastheerspecificiteit die reeds zijn beschreven in de literatuur. Tot slot zetten we nog eens alle pro's en contra's voor een bijzonder rol van het varken als intermediaire gastheer of als mengvat op een rijtje.

In **hoofdstuk 2** werden de fundamentele redenen voor dit onderzoek uiteengezet evenals de vooropgestelde specifieke doelstellingen van dit thesisonderzoek. In de daaropvolgende drie hoofdstukken werden alle onderzoeksresultaten, verkregen binnen dit onderzoek, gerapporteerd.

In **hoofdstuk 3** werd de ontwikkeling van een *ex vivo* systeem beschreven dat bestaat uit respiratoire neusmucosa, trachea, tertiaire bronchiën en longweefsel. Behalve een opvolging van de vitaliteit in de tijd, werd ook de gevoeligheid voor verschillende influenza isolaten getest. De aviaire stammen bleken duidelijk inefficiënte vermeerderaars in de weefsels van respiratoire neusmucosa en trachea, waar dit voor varkensisolaten en humane isolaten niet het geval was. Naarmate men dieper in de luchtwegen afzakte, werd dit onderscheid in virusreplicatie tussen de verschillende virussen kleiner tot nihil. De resultaten verkregen voor virustitratie van het supernatans werden bevestigd via

immunohistochemische kleuringen van geïnfecteerde cellen om te verifiëren dat positieve cellen wel degelijk in het epitheel waren gelokaliseerd. Verrast door de lage vermeerdering van aviaire influenza virussen, werd besloten de distributie van siaalzuurreceptoren opnieuw te bestuderen aan de hand van lectinekleuringen, waarbij verse weefsels werden ingesloten als controles. Deze resultaten bevestigden een beperkte aanwezigheid van aviaire receptoren op de apicale aflijning van de respiratoire neusmucosa, trachea and bronchiën. Ter hoogte van de longen was deze receptor sterker aanwezig. De humane siaalzuurreceptor werd terug gevonden over de volledige lengte van het respiratoir stelsel. Tot slot werden de lectinekleuringen nogmaals herhaald volgens eerder beschreven protocollen om uit te sluiten dat de door ons verkregen resultaten het gevolg waren van verschillen in de toegepaste technieken.

Aan de hand van twee recombinante virussen die onderling uitsluitend verschillen in receptor bindingstropisme ten gevolge van twee mutaties in het HA, werd in **hoofdstuk 4** nagegaan in welke mate een aviair bindingstropisme limiterend kan zijn voor de virusvermeerdering van een influenzavirus bij het varken. Het betrof de via reverse genetics gecreëerde virussen A/Hong Kong/1/68 en zijn aviaire tegenhanger met volgende substituties: L226G en S228Q. In een eerste studie werd de transmissie efficiëntie van beide virussen vergeleken door 6 geïnoculeerde dieren na 2 dagen in contact te brengen met naïeve varkens. Virusexcretie in neusswabs en seroconversies werden opgevolgd bij alle dieren. Alle varkens geïnfecteerd met de humane recombinant kenden een efficiënte nasale secretie resulterend in virus transmissie naar alle contactdieren, reeds vanaf de eerste dag na contact. De varkens geïnoculeerd met de aviaire variant vertoonden een uitgesproken individuele variatie en een beduidend lagere virusexcretie dan de andere groep. Virus werd in dit geval bij geen enkel contactdier geïsoleerd uit de neusswabs. De inefficiënte transmissie van de aviaire variant ging samen met een lagere virusvermeerdering in alle getitreerde weefsels met uitzondering van de olfactorische neusmucosa. Hoofstuk 4.2. spitste zich in meer detail toe op potentiële mechanismen die aan de basis liggen van de lage vermeerdering van de aviaire variant, zowel *in vivo* als *in vitro*. Aan de hand van immunohistochemische kleuringen op respiratoire weefsels van geïnfecteerde dieren werd gepoogd een verschil in celtropisme tussen beide virussen aan te tonen. Omdat hier geen uitgesproken verschil bleek te zijn, werden bijkomend interacties met mucus en surfactant eiwitten bestudeerd evenals een mogelijk onevenwicht tussen HA en NA functies. Voor de interacties met mucus en SP-D werden verschillen opgemerkt.

Tot slot werd in **hoodstuk 5** de transmissie van een andere aviaire A/Hong Kong/1/68 variant bestudeerd met 5 mutaties in het HA. Op deze 5 locaties werden substituties opgemerkt bij overdracht van de aviaire HA naar de mens, maar de functionele betekenis en het belang voor

mammaire adaptatie werden niet eerder bestudeerd. De transmissie resultaten weerspiegelden een ineffeciënte en uitgestelde overdracht van dit virus naar contactdieren. Dit suggereerde dat één of meerdere van deze mutaties bijdragen tot de aanpassng van het virus van een aviaire naar mammaire gastheer. Sequenering van het HA1, geïsoleerd uit neusswabs van contactdierdieren, bracht een bijkomende mutatie aan het licht: D60G. Een preliminaire fenotypische karakterisatie van dit isolaat in NH_4Cl testen, opperde een eventuele betrokkenheid bij de pH-afhankelijke fusieactiviteit van het HA, wat een bijkomende determinerende factor kan zijn voor virustransmissie en gastheer- specificiteit.

In **hoofdstuk 6** werden de resultaten verkregen in deze thesis onderheven aan een kritische discussie en kwamen potentiële toekomstplannen aan bod. Vooreerst werd de optimalisatie van het *ex vivo* systeem besproken in samenhang met virusreplicatie and siaalzuurexpressie. Bijzondere aandacht werd daarbij besteed aan de nadelen van lectinekleuringen en aan de toepasbaarheid van alternatieve technieken. Vervolgens werd de noodzaak van een mammair bindingstropisme voor replicatie en transmissie van influenzavirussen in het varken bediscussieerd evenals een hypothese omtrent de selectieve rol van mucus en surfactant eiwitten. Tot slot werd een potentieel bijkomend mechanisme voor gastheeradaptatie besproken.

CURRICULUM VITAE

Personalia

Sjouke Van Poucke werd geboren te Gent op 29 maart 1981. In 1999 beëindigde zij haar studies secundair onderwijs in de richting Latijn-Wiskunde aan het Koninklijk Lyceum te Gent. Na een verblijf van 1 jaar in Montana, U.S.A. in het kader van een AFS-programma, startte zij haar studies diergeneeskunde aan de UGent in Gent. Daar behaalde zij in 2007 het diploma van dierenarts, optie onderzoek en industrie, met onderscheiding.

In juli 2007 startte zij haar onderzoek naar de gastheerbarrières voor influenzavirussen bij het varken in het laboratorium voor Virologie van de vakgroep Virologie, Parasitologie en Immunologie, Universiteit Gent. Eerst was zij werkzaam als wetenschappelijk medewerker op het Europese FLUPATH project. Vervolgens werd zij assistent aan diezelfde vakgroep. Naast het begeleiden van de practica algemene virologie en het opvolgen van diagnosen, zette zij haar onderzoek verder binnen het FLUPIG project. Sjouke Van Poucke is auteur of co-auteur van meerdere publicaties in internationale wetenschappelijke tijdschriften en nam actief deel aan nationale en internationale congressen.

Publications in international scientific peer-reviewed journals

- Van Poucke S., Uhlendorff J., Wang Z., Billiau V., Nicholls J., Matrosovich M., Van Reeth K. (2012). Effect of receptor specificity of A/Hong Kong/1/68 (H3N2 influenza virus variants on replication and transmission in pigs. Influenza and Other Respiratory Viruses 7: doi: 10.1111/j.1750-2659.2012.00376.x

- Nicholls J.M., Wong L.P.W., Chan R.W.Y., Poon L.L.M., So L.K., Yen H., Fung K., Van Poucke S., Peiris M.J.S. (2012). Detection of highly pathogenic influenza and pandemic influenza virus in formalin fixed tissues by immunohistochemical methods. Journal of Virological Methods 179: 409-413

- De Vleeschauwer A., Van Poucke S., Karasin A., Olsen C., Van Reeth K. (2011). Cross-protection between antigenically distinct H1N1 swine influenza viruses from Europe and North America. Influenza and Other Respiratory Viruses 5: doi: 10.1111/j.1750-2659.2010.00164.

- Van Poucke S., Nicholls J., Nauwynck H.,Van Reeth K. (2010). Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. Virology Journal 7: doi:10.1186/1743-422X-7-38.

- De Vleeschauwer A., Van Poucke S., Braeckmans D., Van Doorsselaere J., Van Reeth K. (2009). Efficient transmission of swine-adapted but not wholly avian influenza viruses between pigs and from pigs to ferrets. Journal of Infectious Diseases 200: 2884-2892.

- Chan R.W.Y., Karamanska R., Van Poucke S, Van Reeth K., Chan W.W., Chan C.W., Dell A., Peiris J.S.M., Stuart H.M., Nicholls J.M. (2013). Infection of swine *ex vivo* tissues with avian viruses and correlation with glycomic analysis. Influenza and Other Respiratory Viruses: conditionally accepted.

Abstracts

- Patrono L.V., Van Poucke S.G., Beato M.S., Terregino C., Van Reeth K., Capua I. (2012). Amino-acid signature L226 in the receptor binding site of the hemagglutinin enhances the replication efficiency of an H9N2 Avian Influenza virus in swine respiratory explants. Proceedings of the 4th Oxford International Influenza Conference, Influenza 2011: Zoonotic Influenza and Human Health, Oxford, UK, p28-29.

- Meyns T., Van Poucke S., Nauwynck H., Loicq M., Joisel F., Vila T. (2012). A case of SMEDI problems due to PCV2 in gilts of a high health breeding herd. Proceedings of the 22nd IPVS Congress: Happy Pigs, Healthy People, Jejun, Korea, p879.

- Van Poucke S., Uhlendorff J., Matrosovich M., Van Reeth K. (2011). Effect of mutations in the receptor-binding site of Hong Kong/1/68 (H3N2) virus on replication and transmission in pigs. Proceedings of the 4th Oxford International Influenza Conference, Influenza 2011: Zoonotic Influenza and Human Health, Oxford, UK, p28-29.

- Van Poucke S.G.M., Nicholls J.M., Nauwynck H.,Van Reeth K. (2009). Time to revise the unique role of the pig as a mixing vessel for influenza A viruses? Summer Course on Infection and Immunity, September, Ghent, Belgium, p18.

- Van Poucke S., Van Reeth K. (2009). Time to revise the unique role of the pig as a "mixing vessel" for influenza A viruses? Proceedings of the 1st Joint Bird Flu Meeting, Barcelona, Spain, p 15.

- Van Reeth K., Van Poucke S., De Vleeschauwer A. (2009). Pigs and pandemic influenza: myths versus facts. Proceedings of the Allan D Leman Swine Conference, Minnesota, United States of America, p23-27.

- Van Poucke S., Van Reeth K. (2008). Time to revise the unique role of the pig as a "mixing vessel" for influenza A viruses? Proceedings of Stress responses in the microbial world, Brussel, Belgium, p114

- De Vleeschauwer A., Braeckmans D., Van Poucke S., Barbé F., Van Reeth K. (2007). Lack of transmission of low pathogenic avian influenza viruses between pigs and from pigs to ferrets. Proceedings of the 3rd ESWI influenza conference, Villamoura, Portugal, p197.

Oral presentations

- Van Poucke S., Uhlendorff J., Matrosovich M., Van Reeth K. (2011). Effect of mutations in the receptor-binding site of Hong Kong/1/68 (H3N2) virus on replication and transmission in pigs. 4th Oxford International Influenza Conference, Influenza 2011: Zoonotic Influenza and Human Health, September, Oxford, UK.

- Van Poucke S.G.M., Nicholls J.M., Nauwynck H.,Van Reeth K. (2009). Time to revise the unique role of the pig as a mixing vessel for influenza A viruses? Summer Course on Infection and Immunity, September, Ghent, Belgium.

- Van Poucke S., Van Reeth K. (2009). Time to revise the unique role of the pig as a "mixing vessel" for influenza A viruses? Proceedings of the 1st Joint Bird Flu Meeting, Barcelona, Spain.

- Van Poucke S., Van Reeth K. (2008). Time to revise the unique role of the pig as a "mixing vessel" for influenza A viruses? Proceedings of Stress responses in the microbial world, Brussel, Belgium.

- Van Poucke S. In vitro cultures of the porcine respiratory tract and their susceptibility to influenza A viruses (2008). ESNIP Minisymposium, June, Ghent, Belgium.

DANKWOORD

Eén ding is me nu wel duidelijk, een doctoraat is veel meer dan wetenschappelijk onderzoek….het is ook de ultieme test als “mens”. Geen van beiden had ik tot een goed einde gebracht zonder de onvoorwaardelijke steun en vriendschap van talloze mensen die een woordje verdienen in dit -naar alle waarschijnlijkheid onvolledige- dankwoord (sorry bij voorbaat aan zij die hun naam niet terug vinden, ik kan enkel hopen dat mijn appreciatie reeds bleek in het verleden…!)

Mijn promotor, Prof. Dr. Van Reeth, wens ik te bedanken voor de hulp bij het schrijven van publicaties, abstracts en deze thesis en om me te betrekken bij de Europese projecten. Dank ook voor de verkregen vrijheid toen dit onderzoek de iets meer moleculaire weg insloeg. Prof. Dr. Nauwynck, jou wil ik bedanken om me de kans te bieden dit onderzoek te verrichten in het Laboratorium voor Virologie, voor de kritische suggesties bij de presentaties en voor je tijd wanneer er nog maar eens twijfel was over die ene positieve cel bij de diagnostiek.

Sincere thanks to the members of the guidance committee Em. Prof. Dr. Pensaert, Prof. Dr. Nicholls and Prof. Dr. Favoreel for the numerous fruitful discussions, the most appreciated suggestions and the genuine interest in this research. I am also most grateful to the members of the reading and examination committee Dr. Matrosovich, Prof. Dr. Saelens, Dr. van den Berg and Prof. Dr. Van den Broeck for their effort and time to help me through the final stages of this PhD.

A special word to you, John for the wonderful hospitality during every stay in Hong Kong, for introducing me to this “mess” called glycobiology, for the endless practical help and most of all for being an inspiration throughout my entire PhD! Mikhail, thank you very much for the good cooperation over the years and the philosophic influenza debates. I could not have hoped for a better challenger ;-) and a better host in Marburg. Debby en het EMC-team, merci voor jullie gastvrijheid tijdens het verblijf in Rotterdam en hopelijk komt er ooit een antwoord op het PVA-Hong Kong vraagstuk!

In die zes jaar op het labo heb ik bijzonder veel toffe collega’s zien komen en gaan, te veel om op te sommen. De fijne sfeer die er heerste heeft ervoor gezorgd dat de late avonden, het vakantie- en weekendwerk ruimschoots werden gecompenseerd met een aangename babbel, cryptosessies en andere hersenbrekers, een kaasschotel, een BBQ, (schrijf)ijsjes, een glaasje wijn of een frisse pint…Dank je collega’s! De blijvers wens ik alle succes met hun onderzoek en zij die reeds andere oorden opzochten veel geluk. During the six years in the lab I saw lots of nice colleagues coming and going, too many to enumerate. Thank you all and good luck with research to the ones still present, lots of happiness to those who have left. Bijzondere dank aan alle influenza-teamleden: Debby, Filip, Annebel, Kalina en Constantinos. Omringend door jullie goede zorgen en adviezen heb ik schoorvoetend mijn eerste stapjes kunnen zetten in het onderzoek. I am very grateful for all the hours we spent discussing our topic or feeding gnoto’s, philosophating about viruses and “real life” (including Greek politics) and the connectedness I was part of. Annebel, ik kan je niet genoeg danken voor het delen van alle dier(soort)experimentele ervaringen en de basis van deze thesis…merci voor alle geduld dat je met me hebt (gehad)! Het ga je goed op je zoektocht ! Zhongfang, brother, the (too short) time we shared the office was very enriching in every way! Thanks for the molecular and moral support, the chinese food and “life is hard” philosophies…I wish you and your family the very best in down under, hope to see you again some day, somewhere! To the new influenza team Yu, Pepe, Karl and Jolien: whatever the research future brings, I wish you all the best of luck! Yu, I am very glad I got

to know you well as a person and as a researcher, you deserve a bright future and I dare you to stack more corns with chop sticks than me ;-)

To the (short-time) visitors Veerle, Elisa, Livia and Francesco it was a blessing to guide you around in the lab, in a small part of the country and of course in the Gent bars ;-) I enjoyed all the good times we shared, hope there are many more to come..! Niemand die me de labo-knepen beter kon bijbrengen dan de vaste waarden Lieve, Nele en Melanie. Zonder jullie expertise en onophoudelijk titreren, MDCK-splitsen, en explanten bereiden kon ik deze thesis onmogelijk verwezenlijken…niks was jullie teveel! Ik heb oprecht genoten van onze samenwerking en koester de uren zij aan zij aan de flow gepaard gaande met een kletske of een welverstaand stilzwijgen, een super dikke merci! Carine en Chantal, dankzij de diagnostiek en de macrofaagspoelingen heb ik ook jullie en niet-influenza virussen beter leren kennen. Bijzondere dank voor de opperbeste samenwerking, de culinaire en toeristische adviezen en de vrolijke noot. Dries en Tim, merci om mij als leek te tolereren en te begeleiden in 't moleculair labo en voor de schitterende ambiance…iets zegt me dat onze (uitgaans)wegen elkaar nog wel eens kruisen ;-) Kevin, I cannot thank you enough for sharing your immunohistochemistry skills, for sacrificing late nights to meet the deadlines and for guiding me through the wonderful Hong Kong culture, cuisine and nature…I really hope to do this for you in return in Europe some day! Christian en Delphine, dank voor het inbedden van de talrijke mini-weefseltjes op sneltempo. Onze dierverzorgers Geert, Zeger en Bart een welgemeende merci voor de talrijke varkens-ophaal-ritten naar Frankrijk (al was ik er die ene keer toch niet echt gerust in Zeger), het bijbrengen van slacht- en stroptechnieken, de dissectiefoto's en het steeds voorzien van mijn "speciale materiaalwensen" (van gridjes tot fretten anesthesie-dozen). Mieke, Ann, Gert(je) en Dirk jullie wil ik hartelijk danken voor de administratieve, boekhoudkundige en IT-gerelateerde bijstand en het eindeloos geduld dat jullie voor ons PhD-ers opbrengen.

Het vaccavet-team wil ik graag bedanken om me met de beide voetjes op de praktijkgrond te houden!

De vrienden van het eerste uur die me ondanks de drukbezette agenda na al die jaren nog hartelijk verwelkomen Donna & Carine, Lieven, Marc, Lies en Matthias….ik beloof het jullie, er volgt compensatietijd! Jack, Linda (aka mom), Mariah and Scot few Belgians can claim they have an American family, thanks to you all I can. No matter how far the distance, I always carry you in my heart. Ketaketemotsen! Jackie, Marie-Jeanne en Michèle zelden zo'n lieve en oprechte levensgenieters ontmoet. Heel erg bedankt dat ik altijd kan binnen waaien, de sofa ter beschikking heb ;-) evenals een woordje advies. Ik hoop nog vele momenten met jullie te mogen delen.

Paul, Sabine, Karel, Lotte, grootouders Gavere en Ouwegem oprechte dank voor het warme onthaal in de familie. De ontelbare keren dat ik de voetjes onder tafel mocht schuiven of een potje meekreeg waren een absolute luxe, de kickersessies en fietstochtjes de ideale manier om de labo-zorgen even volledig te vergeten.

Mama, papa en bomma ik ben jullie bijzonder dankbaar voor alle kansen en de onophoudelijk steun die ik van jullie heb gekregen. Jullie nemen me zoals ik ben (inclusief de mindere kantjes…) en hebben me het nodige (zelf)vertrouwen gegeven! Deze thesis is daarvan het resultaat, hopelijk kunnen jullie er een beetje fier op zijn…..Broer(tje), voor jou moet het nog allemaal beginnen. Weet dat de deur altijd voor je zal open staan.

Bolleke, mijn steun en toeverlaat, geen woorden kunnen beschrijven wat je voor me betekent. Ik kijk bijzonder uit naar onze “nieuwe start” samen en zie je graag!

Sjouke, Gent 2013